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13. ABSTRACT ***(Maximum 200 Words)***

In this report, we present evidence that two novel kinases, *Hunk* (hormonally upregulated *neu*-tumor associated kinase) and *Punc* (pregnancy upregulated novel CaM kinase) isolated in a screen designed to identify protein kinases involved in mammary gland differentiation and carcinogenesis may be playing a role in both processes. *Hunk* and *Punc* are both developmentally regulated with *Hunk* levels highest early in pregnancy and *Punc* expression peaking late in pregnancy. In addition, their punctate spatial expression patterns suggest that *Hunk* and *Punc* may be markers for mammary epithelial cell subtypes. Consistent with their developmentally regulated expression patterns, we provide initial data suggesting that Hunk, in the context of an MMTV transgenic animal, affects normal differentiation of the mammary gland. Data also suggests that *Hunk* and *Punc* may be playing a role in carcinogenesis. Both kinases display an oncogene-restricted expression pattern with *Punc* expressed exclusively in tumor cell lines initiated by *int-2* and *c-myc* and *Hunk* expression restricted to *Neu* and *H-ras* transformed cell lines. Investigation of kinase expression in a panel of human tumor cell lines and in primary human tumors reveals expression of *Hunk* and *Punc* in subsets of cell lines of different tumor types, including several breast cancers. Taken together, we hypothesize that *Hunk* and *Punc* may be kinases in signal transduction cascades involved in both the development and possibly the transformation of the mammary epithelium.

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FOREWORD

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Heather Lorg Gardner 9/30/97

PI - Signature Date

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INTRODUCTION:

A woman's lifetime risk for developing breast cancer is fundamentally related to reproductive events, in particular those events such as pregnancy, which affect the differentiation state of the breast. One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer(1) Similarly, rats or mice that have previously undergone a full-term pregnancy are highly resistant to carcinogen-induced mammary tumorigenesis as compared to age-matched nulliparous controls(2-4). As such, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

In the breast, as in other systems, proto-oncogenes are molecules that normally function to promote cellular differentiation and proliferation, but when mutated or aberrantly expressed can result in hyperplasia, dysplasia and carcinogenesis. Many proto-oncogenes in the breast are growth factors or growth factor receptors which activate their respective signal transduction pathways via activation of the intracellular kinase domain of the receptor. *EGFR* and *ERBB-2/HER-2/neu* are prime examples of receptor tyrosine kinases that are involved in mammary tumorigenesis through amplification and overexpression and are each found to be amplified in approximately 20% of all human breast tumors. These receptor tyrosine kinases are also involved in the normal development of the mammary gland. For example, overexpression of a dominant negative form of ErbB2 in the murine mammary gland results in the failure of the gland to terminally differentiate as evidence by the failure to form distended lobuloalveoli during lactation(5).

PRELIMINARY DATA:

Studies of protein kinases in a variety or model systems have emphasized the importance of protein kinases in the processes of differentiation and proliferation. Therefore, we initiated and carried out a screen designed to identify protein kinases in the breast that were likely to play a role in mammary development and carcinogenesis(6). As a first step in this study, we first identified protein kinases expressed in the mammary gland by employing degenerate PCR to create a protein kinase catalytic domain library. From this screen, 41 kinases were identified including 33 tyrosine and 8 serine/threonine kinases, 3 of which were novel. In order to determine which kinases may be playing a role in mammary development, we used Northern hybridization analysis to screen for kinases with developmentally regulated mRNA expression patterns. While many of the kinases exhibited subtle (less than 2-3 fold) changes in expression, several kinases showed more dramatic changes in expression during specific stages of mammary development.

Two kinases whose expression changes dramatically during the postnatal development of the mammary gland were two of the novel kinases, *Hunk* and *Punc*(7, 8). *Hunk* levels were markedly upregulated early in pregnancy, a period characterized by rapid alveolar proliferation and differentiation. *Punc* was also upregulated early in pregnancy, but in contrast to the return of *Hunk* expression to basal levels by midpregnancy, expression of *Punc* continued to rise throughout pregnancy, peaking just prior to parturition. *Punc* expression, when normalized to actin expression, returns to basal levels during lactation. This pattern of developmental regulation implies that *Hunk* may play a role in mammary gland development early during pregnancy, whereas *Punc* may function later during pregnancy.

In order to determine whether the upregulation of *Hunk* and *Punc* expression occurring during pregnancy reflects global changes in expression throughout the mammary gland, or changes in specific cell types, the spatial patterns of expression of *Hunk* and *Punc* in the mammary gland were determined by *in situ* hybridization(7, 8). Consistent with results from RNase protection analysis, *Hunk* expression is higher at day 7 of pregnancy compared to day 20. The upregulation of *Hunk* observed by RNase protection appears to be the result of dramatic upregulation within a subset of epithelial cells. The same is true for *Punc* expression. Highest expression of *Punc* is observed in the mammary gland just prior to parturition with a subset of cells expressing at high levels. This strikingly punctate expression pattern was not observed for other kinases examined and suggests that *Hunk* and *Punc* are differentially expressed in the mammary gland in distinct epithelial cell subtypes that are differentially regulated during pregnancy.

Transgenic mouse models of human breast cancer have been generated where oncogenes such as *neu, H-ras, int-2* and *c-myc* are overexpressed in the murine mammary gland using the MMTV promoter(9-11). Transgenic mice overexpressing these oncogenes develop mammary tumors. As a second screen designed to identify kinases potentially involved in breast cancer, we determined the expression of each protein kinase in a panel of mammary epithelial tumor cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing either the *neu, c-myc, H-ras*, or *int-2* oncogenes under the control in the MMTV promoter. Expression patterns of the protein kinases varied from no expression to ubiquitous expression, with many kinases being expressed in a heterogeneous pattern among tumor cell lines(6).

Consistent with their epithelial pattern of expression as determined by *in situ* analysis, neither *Hunk* or *Punc* was expressed in the fibroblast cell line, 3T3, suggesting that both kinases may be epithelial specific(7, 8). Moreover, of the 41 kinases examined, only *Hunk* and *Punc* showed an expression pattern that correlated with transformation by a particular oncogene. *Hunk* is expressed in all cell lines derived from *neu* or *H-ras* initiated tumors and is not detected in any of the cell lines derived from *myc* or *int-2* tumors. Lower levels of *Hunk* expression were observed in the non-transformed mammary tumor cell line, NMuMG. Interestingly, *Punc* exhibited an oncogene-restricted expression pattern which was the inverse of that observed for *Hunk*. *Punc* expression was detected in tumor cell lines derived from *int-2* and *c-myc* transgenic animals but not in cell lines derived from *neu* or *H-ras* tumors. These mutually exclusive, oncogene-restricted expression patterns suggest either that expression of *Hunk* and *Punc* are directly regulated by these oncogenes, or that *Hunk* and *Punc* are markers for epithelial cell subsets that are preferentially transformed by a their respective oncogenes.

Taken together, our initial results suggest that we have identified two novel serine/threonine kinases that may play a role in mammary gland development and/or carcinogenesis. *Hunk* and *Punc* expression are each developmentally regulated with *Hunk* expression levels peaking early in pregnancy and *Punc* expression levels peaking late in pregnancy. These kinases are also expressed in mutually exclusive subsets of mammary tumor cell lines with defined initiating events that are relevant to human cancers. In addition, their punctate spatial expression patterns suggest that *Hunk* and *Punc* may be markers for mammary epithelial cell subtypes. As such, we initiated further investigation of the role of *Hunk* and *Punc* in mammary gland development and carcinogenesis. In addition to the data presented above, we summarize below our progress towards completion of the objectives outlined in our proposal and statement of work.

BODY:

Technical Objective I: Isolate and characterize full-length murine cDNA clones for novel protein kinases.

Task1: Months 1-12: Isolate full-length cDNA clones for novel kinases

In order to obtain full-length cDNA clones for *Hunk* and *Punc*, cDNA libraries were screened and the resulting cDNA clones were sequenced on both strands. Consistent with the length of the *Hunk* transcript as determined by Northern analysis, a 5024 nucleotide cDNA encoding *Hunk* has been isolated from a day 14 mouse embryo library (Genbank accession #AF167987)(12). There is a putative initiation codon at nucleotide 72 and an inframe stop at nucleotide 2216 resulting in an open reading frame of 714 amino acids. *In vitro* translation of *Hunk* results in a protein product consistent with the predicted molecular weight of 79.6 KDa which comigrates with endogenous protein as determined by IP/Western analysis using antisera raised against nonoverlapping regions of Hunk.

Three *Punc* cDNA clones ranging from 1455 to 1510 nucleotides were isolated from a murine brain cDNA library (Genbank accession #AF181984)(13). These clones differed only in their 5' UTR sequence, the significance of which has not been determined. Northern analysis of murine brain mRNA reveals a transcript approximately 1.5 kb in length, consistent with the sizes of the cDNA clones isolated. Conceptual translation of the 343 amino acid open reading frame predicts a protein product of 38.6 KDa, consistent with the protein product observed following *in vitro* translation of the *Punc* cDNA.

Task 2: months 1-12: Characterize structure of novel protein kinases.

The full-length *Hunk* cDNA encodes a protein of 714 amino acids with a kinase catalytic domain spanning amino acids 58 through 319 and containing all residues conserved among serine/threonine kinases(12). In addition, a recently described SNF1 homology domain (SNH) is found C-terminal to the catalytic domain(14). Although this domain is conserved among SNF1 family members, its function is unknown. The C-terminal region of *Hunk* does not share significant homology with other known molecules.

The kinase catalytic domain of *Punc* spans amino acids 13 to 272 and contains all amino acids conserved among serine/threonine kinases(13, 15) . C-terminal to the kinase domain is a regulatory domain common to members of the CaM kinase family which is comprised of overlapping autoinhibitory and Ca^{2+}/calmodulin (CaM) binding domains(16).

Multiple sequence alignment software was used to determine the relationship within kinase catalytic subdomains I though XI between *Hunk*, *Punc* and other serine/threonine kinases. Interestingly, *Hunk* and *Punc* are each found within the calcium regulated, serine/threonine kinase superfamily group(17). This group contains members of both the SNF1 and CaM kinase families.

Although *Hunk* is most closely related to SNF1 kinase family, it does not fall within one of the subfamilies within this group(12). Two main branches exist within the SNF1 family tree. One contains *SNF1* and its plant homologues, *NPK5, AKin10, BKIN12*, and *Rkin1* as well as the *SNF1* mammalian homologue *AMPK*. The second main branch of the SNF1 family contains a group of newly described kinases, *MARK1, MARK2, C-TAK1/p78, Msk*, and *par-1*, which have been implicated both in development and in cell cycle regulation. Based upon homology within the kinase domain, *Hunk* appears to define a new class of SNF1 kinases, members of which have been implicated both in development and carcinogenesis in higher eukaryotes.

Within the kinase domain, *Punc* is 70% identical to *CaMKI* and approximately 50% identical to *CaMK II* and *IV*(13). The CaM kinase family includes smooth muscle myosin light chain kinase (MLCK), Phosphorylase kinase, DAPK ,and multifunctional CaM kinases including CaMKI, CaMKIV, and the well described CaMKII subfamily(18). Although *Punc* is most closely related to *CaMKI*, the homology between *Punc* and *CaMKI* is lower than homology between the α,β,γ,and δ isoforms of the CaMKII subfamily. Homology between *Punc* and other CaM kinase family members is also high in the regulatory domain which consists of the autoinhibitory and CaM binding regions. Consistent with the primary amino acid sequence of other CaM kinases, *Punc* contains unique regions at both the extreme N- and C-termini.

Technical Objective II: Characterize temporal and spatial expression patterns

Task 1: months 6-24: Characterize the temporal expression pattern of novel kinases during mammary gland development

Task 2: months 1-24: Characterize the spatial expression pattern of novel kinases in the murine mammary gland during development.

The temporal and spatial mRNA expression patterns of *Hunk* and *Punc* during murine mammary gland development are described in the preliminary studies section above.

Technical Objective III: Determine the functional roles of *Hunk* and *Punc*

Task1: months 12-48: Determine the role of novel kinases in differentiation.

To test the hypothesis that *Hunk* and *Punc* are involved in the differentiation of the mammary gland during pregnancy, transgenic mice were generated to overexpress each kinase in the mammary gland using the hormonally responsive, mammary specific promoter from the mouse mammary tumor virus (MMTV) LTR. As described above, *Hunk* is upregulated early in pregnancy but returns to basal levels by mid-pregnancy. If regulated expression of *Hunk* plays a role in mammary gland development early in pregnancy, then aberrant overexpression of *Hunk* later in pregnancy may affect the ability of the gland to properly differentiate. Similarly, although *Punc* expression is upregulated later in pregnancy, endogenous levels return to basal expression during lactation. Since MMTV driven transcription peaks during lactation, MMTV-*Hunk* and MMTV-*Punc* transgenic mice would be predicted to have altered developmental patterns

of expression. This altered regulation will help address whether overexpression of either kinase during pregnancy or lactation disrupts normal differentiation of the mammary gland.

Mammary gland-specific transgene expression was investigated in MMTV-*Hunk* and MMTV-*Punc* transgenic animals. Organs were harvested from a virgin animals and transgene expression was determined by RNase protection analysis. Highest transgene expression was detected in mammary glands from MHK3 (MMTV-*Hunk*) and MPK6 (MMTV-*Punc*) transgenic female mice(7, 8).

Interestingly, most pups from MHK3 transgene-expressing mothers died within 2 days of birth suggesting the transgenic females have a defect in their ability to lactate. To determine the effect of Hunk overexpression on mammary development, MHK3 transgenic females were sacrificed at different stages of development including puberty, pregnancy, lactation and post-lactational regression. Whole mounts and H&E stained sections were used to screen for abnormalities in the mammary gland. As predicted, the most dramatic developmental abnormality in MHK3 animals occurs during lactation. In addition, expression of milk-protein genes was significantly lower in MHK3 transgene-expressing mice compared to both wild-type and transgene non-expressing animals during late pregnancy and lactation. In aggregate, these observations suggest that exogenous overexpression of *Hunk* prevents terminal differentiation of the mammary gland during lactation.

MMTV-*Punc* mice are in the process of being analyzed.

Task 2: months 12-48: Define the role of the novel kinases in proliferation.

Endogenous *Punc* RNA is upregulated in confluent cells when compared to actively proliferating cells suggesting that *Punc* may be playing a role in cell proliferation. However, *Punc* expression is not affected when cells are forced into G_0 by serum starvation (data not shown). Clones stably transfected with *Hunk* and *Punc* are being analyzed to determine the level of transgene overexpression and the effect of kinase overexpression on proliferation in tissue culture cells. In combination with the analysis of transgenic animals, these experiments will be important in determining whether or not *Hunk* and *Punc* directly affect cellular proliferation.

Task 3: months 18-48: Determine the role of Hunk and Punc in carcinogenesis.

As described above, *Hunk* and *Punc* are expressed in a mutually exclusive set of murine epithelial tumor cell lines with defined initiating events. This observation suggests either that *Hunk* and *Punc* are downstream of their respective oncogenes or that *Neu* and *myc* preferentially transform a cell type that is marked by *Hunk* and *Punc* expression respectively.

In order to determine the role of *Hunk* and *Punc* in human cancer, we isolated cDNAs encoding the human homologues of these kinases(19, 20). Amino acid conservation in the coding region between mouse and human Hunk is greater than 90% identical. While a cDNA clone encoding the entire Punc ORF has not been isolated, partial cDNA sequence has been obtained and has allowed for the generation of nucleotide probes specific to *Punc* and together, these reagents have allowed us to begin to investigate the role of *Hunk* and *Punc* in human cancers.

Based upon expression of *Hunk* and *Punc* in the murine tumor cell lines, we hypothesize that these kinases may also be differentially regulated in human tumor cell lines. To address this question, RNA was harvested from approximately 60 human tumor cell lines representing different types of human cancers. RNase protection analysis reveals that both *Hunk* and *Punc* are expressed in a subset of cell lines from a variety of tumor types, although these subsets are not mutually exclusive as was observed for the murine tumor cell lines. Similarly, in RNA samples from a variety of primary human tumors, including breast, *Hunk* and *Punc* are each expressed in a subset of tumors. Interestingly, *Hunk* expression in colon and ovarian tumors is higher than in normal tissue suggesting that *Hunk* may be actively involved in the transformation process. We are currently investigating the *neu* and *c-myc* status of these samples to determine if *Hunk* and *Punc* expression correlates with oncogene expression in human cancers in a manner consistent with results obtained from murine tumor cell lines.

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APPENDIX

The funding period of the predoctoral training award described in this report expired September 14, 1999. All specific aims of the grant are near or at completion. Ms. Gardner's anticipated thesis defense date will be in January, 2000. This predoctoral training award provided funding for her doctoral thesis work, and has resulted in several publications as listed below.

Key research accomplishments:

Ph.D. degree, expected February, 2000:
Dept. of Molecular Biology, Biomedical Graduate Studies,
University of Pennsylvania School of Medicine, Philadelphia, PA
Thesis Advisor: Lewis A. Chodosh, M.D., Ph.D.
Ph.D. Thesis: *The Role of Two Novel Kinases in Mammary Gland Development and Carcinogenesis.*

Oral Presentation - *The 51st Annual Symposium on Fundamental Cancer Research*, Houston, Texas. October, 1998.

Travel Fellowship Award - *The 51st Annual Symposium on Fundamental Cancer Research*, Houston, Texas. October, 1998.

Abstract - "Two Novel Protein Kinases in Mammary Gland Develoment and Carcinogenesis." *The 51st Annual Symposium on Fundamental Cancer Research*, Houston, Texas. October, 1998.

Publications:

Chodosh, L. A., D'Cruz, C. M., Gardner, H. P., Ha, S. I., Marquis, S. T., Rajan, J. V., Stairs, D. B., Wang, J. Y., and Wang, M. Mammary gland development, reproductive history, and breast cancer risk, *Cancer Research, 59*, 1765-1771s (1999).

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Manuscripts submitted: (see attached)

Chodosh, L. A., Gardner, H. P., Rajan, J. V., Stairs, D. B., Marquis, S. T., and Leder, P. *Protein kinase expression during murine mammary gland development.*

Gardner, H. P., Wertheim, G. W., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T., and Chodosh, L. A. *Cloning and characterization of Hunk, a novel mammalian SNF1-related protein kinase.*

Gardner, H. P., Rajan, J., Ha, S., Copeland, N., Gilbert, D., Jenkins, N., Marquis, S., and Chodosh, L. *Cloning, characterization, and chromosomal localization of Punc, a calcium/calmodulin-dependent protein kinase.*

Gardner, H. P., Belka, G., Wertheim, G., Ha, S. I., Marquis, S., and Chodosh, L. A. *Developmental role of Hunk in mediating pregnancy-induced changes in the mammary gland.*

Manuscripts in preparation:

Gardner, H. P., Ha, S. I., and Chodosh, L. A. *Differentiation-dependent expression of a novel calcium/calmodulin-dependent protein kinase, Punc, in the murine breast.*

Gardner, H. P., Hartman, J. L., Reynolds, C. A., Ha, S. I., and Chodosh, L. A. *The human homologue of Hunk, a developmentally regulated murine kinase, is overexpressed in subsets of human cancers.*

Gardner, H. P., Hartman, J. L., Mintzer, K. A., Ha, S. I., Reynolds, C. A., and Chodosh, L. A. *Expression of the human homologue of Punc is restricted to subsets of human tumors.*

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Two Novel Kinases in Mammary Gland Development and Carcinogenesis

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Studies in a variety of biological systems have emphasized the importance of protein kinases in differentiation, development, and carcinogenesis. Therefore, we have initiated a systematic study of the role of protein kinases in mammary gland development and carcinogenesis. A screen designed to identify kinases expressed in the murine mammary gland has yielded 43 protein kinases, including 3 kinases that appear to be novel. The expression pattern for each of the kinases identified has been determined during mammary gland development and in a panel of both normal and transformed mammary epithelial cell lines. Interestingly, most of the kinases, including two of the novel kinases, Hunk and Punc, show a significant degree of regulation during mammary gland development. More striking, however, is the observation that Hunk and Punc show a mutually exclusive pattern of expression in a subset of mammary tumor cell lines derived from tumors arising in different transgenic mice. Given the unique expression patterns of these novel kinases, we have proceeded with further characterization of Hunk and Punc.

Full-length cDNA clones for Hunk and Punc were obtained by cDNA library screening. Punc shares 50% to 70% homology in the kinase domain with members of the calcium/calmodulin-dependent family of serine-threonine kinases. Hunk is most homologous to the SNF1 family of serine-threonine kinases within the kinase domain yet appears to be unique given the low homology outside the catalytic domain. Using RNase protection assay, northern analysis, and in situ hybridization, we have determined the spatial and temporal expression pattern of both kinases during mammary gland development in a variety of adult tissues and in the developing mouse embryo. Our data suggest that Hunk may be involved in the initial differentiation and proliferation of the mammary gland occurring early in pregnancy, while Punc may play a role late in pregnancy.

Additional studies are under way to determine the biological role of each of these molecules in mammary gland differentiation. Transgenic mouse models have been generated and are being studied to determine the effect of Hunk and Punc overexpression in the mammary gland. Preliminary results indicate that Hunk, which is normally upregulated early in pregnancy, may delay or prevent terminal differentiation of the mammary gland during lactation. In addition, studies are under way to investigate the role of these kinases in the etiology of human breast cancer and their possible utility as prognostic markers.

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Mammary Gland Development, Reproductive History, and Breast Cancer Risk[1]

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Abstract

The observation that normal pathways of differentiation and development are invariably altered during the process of carcinogenesis implies an intrinsic relationship between these processes. This relationship is particularly evident in the breast, as exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events. Understanding the mechanisms by which normal developmental events alter breast cancer risk is a central focus of our laboratory. Herein, we describe three approaches being taken in our laboratory toward defining the molecular basis of this relationship. These include: determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2*, in the normal differentiation and development of the breast; studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development; and defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. This implies an intrinsic relationship between these processes. The existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy epitomizes this relationship. The recognition that breast cancer risk is determined in part by the same reproductive endocrine events that drive mammary gland development argues that mammary gland development and mammary carcinogenesis are fundamentally related.

One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life (*i.e.*, early parity) have a significantly reduced lifetime risk of breast cancer (1). The magnitude of this parity-induced protection against breast cancer is similar in many countries and ethnic groups, regardless of endemic incidence. This suggests that protection results from an intrinsic effect of parity on the biology of the breast rather than from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. This conclusion is bolstered by the observation that rats that have previously undergone a full-term pregnancy are resistant to the induction of breast cancer by administration of the carcinogen DMBA,[3] as compared to age-matched nulliparous controls (2, 3). Therefore, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

A second illustration of this principle comes from the observation that breast cancer risk attributable to exposure to ionizing radiation is a function of age at the time of exposure. Specifically, studies of women who received mantle irradiation for Hodgkin's disease or who underwent repeated fluoroscopy in the course of treatment for tuberculosis have demonstrated that breast cancer risk is significantly greater in women who were exposed to ionizing radiation during adolescence as compared to women exposed at later ages (4, 5). Analogously, nulliparous rats fed DMBA are more likely to develop breast cancer if they are exposed during puberty rather than as mature adults (6). Interestingly, epidemiological studies suggest that the increased susceptibility of the immature human breast to early events in carcinogenesis may occur prior to as well as during puberty. Studies of survivors from Hiroshima and Nagasaki indicate that the greatest increase in breast cancer risk occurred in women who were less than 10 years old at the time of exposure (7). The observed increase in breast cancer incidence in women irradiated during the first year of life for presumed thymic enlargement is perhaps an even more impressive illustration of this principle, given the rudimentary state of the mammary gland at this age (8). Together, these studies suggest that the susceptibility of the mammary gland to carcinogenesis is related to the gland's developmental state at the time of exposure to mutagenic agents and that the immature breast is particularly susceptible to early events in carcinogenesis.

Understanding the molecular and cellular mechanisms by which normal developmental events alter breast cancer risk is a central goal of our laboratory. We believe that achieving this goal requires a more complete understanding of the manner in which hormones and reproductive history alter subpopulations of epithelial cell types present in the breast and of the roles played by key regulatory molecules in these processes. Toward this end, we are currently focusing on: (*a*) determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2*, in the normal differentiation and development of the breast; (*b*) studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development and carcinogenesis; and (*c*) defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Tumor Suppressor Genes: *BRCA1* and *BRCA2*

The epidemiological relationship between development and carcinogenesis is illustrated on a molecular and mechanistic level by the existence and function of tumor suppressor genes such as *p53*, the Wilms' tumor gene (*WT1*), and the retinoblastoma susceptibility gene (*RB*). Germ-line mutations in these genes are associated with inherited cancer predisposition syndromes (9). The cloning and analysis of several tumor suppressor genes has revealed that they frequently

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; EGFR, epidermal growth factor receptor.

encode proteins that act as negative regulators of cell proliferation, exert cell cycle checkpoint control function, or maintain genome integrity (10, 11). In addition, the targeted deletion of these genes in mice frequently results not only in increased susceptibility to cancer but also in abnormalities in proliferation, apoptosis, differentiation, and development (10, 12). As such, one approach to elucidating the relationship between mammary gland development and carcinogenesis is to determine the function of tumor suppressor genes known to be involved in the pathogenesis of breast cancer.

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germ-line mutations in autosomal dominant susceptibility genes (13, 14). Over the past 4 years, several of these breast cancer susceptibility genes have been isolated by positional cloning, including *BRCA1* and *BRCA2* (15–19). Tumors arising in patients with germ-line mutations in either *BRCA1* or *BRCA2* typically display loss of the corresponding wild-type allele, suggesting that *BRCA1* and *BRCA2* are tumor suppressor genes (20–22). Interestingly, *BRCA1* and *BRCA2* mutations have not been identified in sporadic breast cancers, despite the fact that 25–30% of sporadic breast cancers show loss of heterozygosity at these loci (16, 23–26). This raises the intriguing possibility that the normal functions of these genes are temporally and/or developmentally restricted.

Recently, important clues to BRCA1 and BRCA2 function have come from biochemical studies demonstrating that treatment of cells with a variety of DNA-damaging agents leads to the rapid phosphorylation of BRCA1 (27, 28). Moreover, both BRCA1 and BRCA2 have been shown to directly or indirectly bind to RAD51, a homologue of RecA that has been implicated in DNA repair and recombination (29–32). These and other observations have led to the hypothesis that BRCA1 and BRCA2 are involved in the cellular response to DNA damage. Consistent with this hypothesis, embryonic cells from mice homozygous for mutations in the *Brca2* locus have an increased sensitivity to DNA-damaging agents (30, 33, 34). It is interesting to speculate that the developmental regulation of *BRCA1* and *BRCA2* expression or function may contribute to the age-dependent susceptibility of the breast to ionizing radiation-induced carcinogenesis described above.

The markedly elevated risk of breast cancer observed in women carrying germ-line mutations in *BRCA1* and *BRCA2* strongly suggests that these gene are critical for the properly regulated growth of mammary epithelial cells. As a first step toward understanding the developmental role of *BRCA1* and *BRCA2*, we have analyzed the spatial and temporal expression of the murine homologues of these genes during embryogenesis, in the mammary gland during postnatal development, and in adult tissues (35, 36). These studies reveal that expression of both *Brca1* and *Brca2* are tightly regulated during mammary gland development. For example, *Brca1* and *Brca2* expression levels in the mammary glands of adolescent female mice undergoing ductal morphogenesis are significantly higher than those found in the mammary glands of mature females in whom ductal morphogenesis has been completed (35, 36). This temporal pattern of expression is explained in part by the observation that *Brca1* and *Brca2* are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation (35–37). *Brca1* and *Brca2* mRNA levels are also markedly up-regulated in the mammary gland early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (35–38). This up-regulation of *Brca1* and *Brca2* expression occurs preferentially in developing alveoli as compared to adjacent epithelial ducts, consistent with patterns of proliferation (35, 36). Indeed, at virtually all stages of development, *Brca1* and *Brca2* expression are restricted to cellular compartments actively involved in proliferation and differentiation. These patterns of expression suggest that these tumor suppressor genes may play a role in the normal development of the breast and other tissues.

The spatial and temporal patterns of *Brca1* and *Brca2* expression during development likely reflect the fact that expression of these genes is tightly regulated as a function of proliferation. We have shown that *Brca1* and *Brca2* mRNA levels are high in exponentially growing cells and low in quiescent cells (39). During progression through the cell cycle, *Brca1* and *Brca2* mRNA levels increase during G_1 and attain maximal levels at the G_1-S transition (39). Similar observations have been made for human BRCA1 and BRCA2 at both the mRNA and protein levels (39–46). These findings clearly demonstrate that proliferative stimuli modulate the expression of these genes. Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, the expression of these genes also appears to be influenced by factors other than proliferation. For example, we have shown that *Brca1* and *Brca2* mRNA levels are coordinately up-regulated in postconfluent HC11 mammary epithelial cells during differentiation as well as following treatment with insulin and glucocorticoids (39). *Brca1* and *Brca2* expression increase in this setting to levels as high as those found in actively proliferating cells, despite the fact that cellular proliferation rates remain low under these experimental conditions. Together, these observations imply that *Brca1* and *Brca2* may be involved in the processes of proliferation and differentiation in the breast.

A particularly intriguing finding of our studies has been the striking degree to which *Brca1* and *Brca2* are temporally and spatially coexpressed at the mRNA level (36). We have found that *Brca1* and *Brca2* are expressed at similar levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, the developmental expression patterns of these two putative tumor suppressor genes are essentially identical during embryogenesis and in multiple tissues of the adult. This similarity is particularly evident during postnatal mammary gland development as *Brca1* and *Brca2* expression are each up-regulated during puberty and pregnancy. The coordinate induction of these genes in proliferating and differentiating mammary epithelial cells *in vitro* may provide a cellular basis for this similarity (39). These findings suggest that similar pathways and stimuli regulate the expression of *Brca1* and *Brca2* in multiple cell types. Taken together with the fact that inherited mutations in either *BRCA1* or *BRCA2* predispose mammary epithelial cells to transformation, the striking similarities in *Brca1* and *Brca2* expression patterns formed the initial basis for speculation that these genes may function in overlapping pathways and may even directly interact.

As alluded to above, no somatic mutations have been identified in *BRCA1* or *BRCA2* in sporadic breast cancers. This puzzling observation could be explained if the function of these cancer susceptibility genes in the mammary gland were restricted to specific developmental stages, as might be suggested by the tightly regulated expression that these molecules exhibit during mammary gland development. Similarly, in light of the proposed relationship between normal mammary gland development and reproductive risk factors for breast cancer, it is interesting to note that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy because these stages of development are each associated with increases in cellular proliferation as well as increases in breast cancer risk. Potentially, the induction of *Brca1* and *Brca2* expression during these developmental stages may be a protective response to proliferation or to DNA damage that accompanies proliferation, as suggested by the observation that *Rad51* is also up-regulated in proliferating cells (35, 47).

Our laboratory has chosen to focus on understanding BRCA1 and BRCA2 function in mammary epithelial cells because considerably

less is known about their function in this context and because breast cancer is the most important clinical phenotype associated with germ-line mutations in these genes. Specifically, we are interested in those aspects of mammary gland biology responsible for the observation that women carrying germ-line mutations in *BRCA1* and *BRCA2* preferentially develop cancer of the breast. Because this may ultimately relate to mammary-specific functions of these molecules, a complete understanding of the role played by these genes in breast cancer susceptibility will almost certainly require that their functions be studied directly in the mammary epithelium. As such, we are analyzing the impact of altering *BRCA1* and *BRCA2* expression levels on proliferation, differentiation, and DNA repair in the mammary epithelium using *in vivo* and *in vitro* model systems. These studies may provide insight into mechanisms of growth control and DNA damage response in normal mammary epithelial cells as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

Novel Protein Kinases

A second approach to investigating the relationship between development and carcinogenesis in the breast is to study members of a family of regulatory proteins that are typically involved in differentiation, development, and carcinogenesis. Analysis of these processes in a variety of model systems has underscored the key role frequently played by protein kinases. Many protein kinases function as intermediates in mitogenic signal transduction pathways or encode growth factor receptors whose overexpression, aberrant expression, or mutation to ligand-independent activated forms results in transformation. Several members of the protein kinase family have been shown to be involved in the development of breast cancer both in humans and in rodent model systems including the epidermal growth factor receptor, the insulin-like growth factor-I receptor, the fibroblast growth factor receptor family, HER2/Neu, Met, and Src. For instance, amplification and overexpression of *HER2/Neu* and *EGFR* have each been correlated with aggressive tumor phenotype and poor clinical prognosis. Similarly, overexpression of certain protein kinases or of their ligands in transgenic animals results in malignant transformation of the mammary epithelium. To date, however, evidence for a causal role of protein kinases in the initiation and progression of breast cancer exists for only a few members of this family of proteins. For this reason, we embarked on a screen designed to identify tyrosine kinases and serine-threonine kinases expressed in the murine breast during normal development and in breast cancer.

First-strand cDNA was prepared from mRNA isolated either from mammary glands of mice at specific developmental stages or from a series of mammary epithelial cell lines derived from breast tumors that arose in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes (48–50). Degenerate PCR was used to amplify kinase catalytic subdomains VI–IX, and the resulting cDNA clones were screened to identify those harboring catalytic domain fragments of protein kinases (51–53). This screen identified 41 kinases: 33 tyrosine kinases and 8 serine-threonine kinases, 3 of which are novel.[4] We have characterized the temporal and spatial expression of these kinases during mammary gland development as well as in a panel of mammary epithelial cell lines derived from breast tumors arising in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes.[4] This analysis has revealed that many of these kinases are preferentially expressed in the breast during specific stages of puberty, pregnancy, lactation, and postlactational regression.

Our laboratory has subsequently focused on the function of three novel serine-threonine kinases identified in our screen: *Hunk*, *Punc*, and *Krct*. The novel protein kinase, *Hunk*, was initially isolated from a mammary epithelial cell line derived from a breast tumor that arose in a transgenic mouse expressing the *neu* oncogene (54).[4,5] Analysis of sequence homology within a portion of the catalytic domain of *Hunk* suggests that it is a serine/threonine kinase with highest homology to the *SNF1* kinase family. The novel protein kinase, *Punc*, was initially isolated from the mammary glands of mice undergoing early postlactational regression.[4,6] The catalytic domain of *Punc* is 60% identical at the amino acid level to calcium/calmodulin-dependent protein kinase I and shares a lower homology with other members of the calcium/calmodulin-dependent kinase family (55).[6] *Krct* appears to represent a new family of mammalian protein kinases and is most closely related to a protein kinase recently identified by the yeast genome project that does not fall into any of the families of protein kinases previously identified in yeast (54).

Hunk and *Punc* appear to be particularly relevant to studies of the relationship between mammary gland development and carcinogenesis by virtue of their patterns of expression.[7,8] Specifically, *Hunk* is expressed at low levels in the mammary glands of immature and mature virgin animals and undergoes a dramatic up-regulation of expression during early pregnancy. *Hunk* expression rapidly drops to basal levels by midpregnancy and decreases further during lactation and early postlactational regression. Like *Hunk*, *Punc* expression is also up-regulated in the mammary epithelium during pregnancy. However, unlike *Hunk*, maximum levels of *Punc* expression occur late in pregnancy just prior to parturition.

To determine whether the developmental changes in *Hunk* and *Punc* expression observed during pregnancy represent global changes in expression occurring throughout the mammary gland or changes in the abundance of an expressing subpopulation of cells, we have defined the spatial pattern of expression of these kinases.[7,8] This was of particular interest because the expression of several protein kinases has been shown to be cell lineage restricted, thereby permitting their use as markers for biologically interesting subpopulations of cells. Examination of the spatial pattern of *Hunk* and *Punc* expression revealed that throughout the course of mammary development both kinases are expressed predominantly in the mammary epithelium. Interestingly, the expression of each of these kinases in the mammary epithelium is strikingly heterogeneous, with the greatest number of *Hunk*-expressing cells being observed at day 7 of pregnancy and the greatest number of *Punc*-expressing cells being observed at day 20 of pregnancy. This pattern of expression does not appear to be due to the heterogeneous distribution of cells through the cell cycle. Analogously, studies of the expression of these kinases in a variety of other tissues suggest that *Hunk* and *Punc* expression may also identify subsets of cells in other organs besides the breast. These observations suggest that *Hunk* and *Punc* are differentially expressed in distinct

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epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

To further investigate this hypothesis, we have examined *Hunk* and *Punc* expression in a panel of mammary epithelial cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing the *neu*, c-*myc*, H-*ras*, or *int2* oncogenes.[7,8] Surprisingly, all eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes were found to express high levels of *Hunk* mRNA, whereas none of the seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed detectable levels of *Hunk*. Conversely, all seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed *Punc* mRNA, whereas none of the eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes expressed detectable levels of *Punc*. In each case, kinase expression levels observed in tumor cells were significantly higher than those observed in nontransformed mammary epithelial cells.

The heterogeneous spatial patterns of *Hunk* and *Punc* expression in the breast, along with the mutually exclusive patterns of expression of these two kinases in transgenic mammary epithelial cell lines, suggest that these novel serine-threonine kinases may be differentially expressed in distinct mammary epithelial cell subtypes that are themselves differentially regulated during pregnancy. The observation that *Hunk* and *Punc* are overexpressed in cell lines derived from breast cancers induced by the *neu* or c-*myc* oncogenes, respectively, suggests either that *Hunk* and *Punc* are downstream targets of the *neu* and c-*myc* oncogenes or that these kinases identify epithelial cell subtypes that are preferentially transformed either by *neu* or c-*myc*.

Each of these hypotheses is based on our observations suggesting that the normal mammary epithelium appears to be composed of distinct *Hunk*- and *Punc*-expressing cell types. The first hypothesis postulates that *Hunk* mRNA expression is activated by the *neu* and/or H-*ras* pathways, whereas *Punc* mRNA expression is activated by the c-*myc* and/or *int2* pathways. In this model, *neu* (or c-*myc*) transgene expression in the mammary epithelium induces *Hunk* (or *Punc*) expression in all mammary epithelial cell types that express the transgene. As a consequence, tumors that arise from the epithelium display the same differential pattern of expression exhibited by the parental normal transgenic mammary epithelium. The second hypothesis postulates that *neu* and c-*myc* preferentially transform two different mammary epithelial cell types, one of which (in the case of *neu*) is marked by *Hunk* expression and the other of which (in the case of *c-myc*) is marked by *Punc* expression. In this model, overexpression of *Hunk* in *neu*-induced tumors reflects the selection and outgrowth of an *Hunk*-expressing epithelial cell subtype that otherwise represents a minor fraction of cells in the normal mammary epithelium. That is, *Hunk* and *Punc* expression may be restricted to distinct epithelial cell subtypes that are preferentially transformed by these oncogenes.

Our data suggest that the novel serine/threonine kinases identified in our laboratory may serve as markers for biologically interesting subpopulations of epithelial cells in the breast that are relevant both to development and carcinogenesis. Current work in our laboratory on *Hunk*, *Punc*, and *Krct* focuses on placing these kinases in known or novel signal transduction pathways and on determining their role in mammary development and carcinogenesis using transgenic and knockout animal models as well as tissue culture model systems. In addition, we have cloned the human homologues for each of these genes and are currently determining whether *Hunk*, *Punc*, and *Krct* are mutated, amplified, or overexpressed in human tumors or tumor cell lines.

Parity-induced Changes in the Breast

A third approach that our laboratory is taking to explore the relationship between development and carcinogenesis in the breast is to focus on the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk. Epidemiological studies have consistently shown that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer (1, 56–64). This association is independent of parity (*i.e.*, number of live births). In contrast, women who undergo their first full-term pregnancy after the age of 30–35 years appear to have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence *per se*. These observations imply that an early first full-term pregnancy results in a change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Because aborted pregnancies are not associated with a decreased risk for breast cancer, it has been hypothesized that the protective effect of parity requires attaining the terminally differentiated state of lactation (2, 3, 6, 59, 65–71). Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. In principle, the protective effect of early first childbirth could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of a subpopulation of target cells during postlactational regression or from a permanent systemic endocrine change affecting the breast in such a way as to reduce the risk of carcinogenesis. Clearly, a more thorough elucidation of the molecular and cellular changes that take place in the breast as a result of parity will be required to fully understand this phenomenon.

The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that, although it is now possible by genetic means to identify women who are at elevated risk for developing breast cancer, interventions between the extremes of more frequent mammographic screening and prophylactic bilateral mastectomy are only now beginning to be considered. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent a feasible alternative. It is to this end that both early first full-term pregnancy and early menopause have been proposed as logical paradigms on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by current ignorance regarding the mechanism by which reproductive history alters breast cancer risk. As such, the rational design of hormonal chemoprevention regimens would benefit from a better understanding of the influence of development on breast cancer risk. An additional stumbling block in the development of chemoprevention regimens aimed at reducing breast cancer risk has been the prolonged and costly clinical trials required to determine the efficacy of these regimens due to reliance on the development of breast cancer as a clinical end point (72–75). As such, the identification and use of intermediate molecular end points that accurately identify changes in the breast associated with changes in breast cancer risk would facilitate the development of such chemopreventive regimens. To this end, we have chosen to exploit the relationship between development and carcinogenesis in the breast to generate rational and biologically plausible candidate surrogate end point biomarkers.

The mechanism of parity-induced protection against breast cancer

is likely to involve complex genetic and epigenetic processes that may be influenced by reproductive endocrine variables as well as by inherited genotypes. In this context, it is useful to analyze complex processes such as this in model systems that recapitulate relevant epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue. The use of animal models to study the impact of mammary gland development on breast cancer risk is facilitated by the fact that the structure, function, and developmental stages through which the mammary gland passes are similar in humans and in rodents (76, 77). Administration of the carcinogen DMBA to nulliparous Sprague Dawley rats induces mammary adenocarcinomas that are hormone dependent and histologically similar to human breast tumors. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration, as compared with age-matched nulliparous controls (2, 6, 78–83).

Paralleling these functional differences, there are also marked morphological differences between the adult nulliparous mammary gland and the mammary glands of age-matched parous littermates that have undergone a single cycle of pregnancy, lactation, and regression. These parity-induced morphological changes are permanent because nulliparous and parous glands may be distinguished easily even after 1 year of postlactational regression (3).[9] Similar morphological changes are also seen in mice and in rats and are analogous to those reported in the parous human breast (70, 77). These observations support the hypothesis that parity results not only in a permanent change in the functional state of the breast (*i.e.*, susceptibility to carcinogenesis) but also in permanent structural changes in the breast. Finally, the fact that the Sprague Dawley DMBA model system mirrors complex epidemiological phenomena observed in humans, and that numerous molecules believed to play important roles in the pathogenesis of human breast cancer have similar effects in rodents, suggests that rodent model systems such as this can be a valuable tool for understanding fundamental aspects of mammary gland biology and breast cancer etiology.

We hypothesize that understanding the impact of parity on breast cancer risk will require a thorough understanding of the manner in which reproductive history affects subpopulations of cell types present in the breast. To address this hypothesis, we are using rodent model systems to identify and evaluate genes that are differentially expressed in the breast as a function of parity. Candidate genes that are specifically expressed in either the parous or the nulliparous rodent breast are being isolated and identified using a variety of approaches. These differentially expressed genes are being used as biomarkers for the cellular and molecular changes that occur in the breast as a result of an early first full-term pregnancy to define the impact of early parity on the development and differentiation of specific cell types in the breast. Finally, biomarkers that are found to be biologically informative in the rodent model system are being tested for their ability to detect parity-associated changes in histologically normal breast tissue obtained from nulliparous and parous women with known reproductive history and hormone exposures. The level and spatial pattern of expression of each of these candidate biomarkers is being analyzed in human tissue and evaluated with respect to parity as well as other parameters of reproductive endocrine history, such as age, age at first full-term pregnancy, menopausal status, and exogenous hormone use. These studies will determine whether candidate biomarkers characterized in rodent model systems can specifically detect parity-induced changes in the human breast.

To date, this approach has yielded a variety of genes that are expressed at higher levels in the mammary glands of parous animals as compared with age-matched virgin controls, confirming the utility of this approach for isolating genes that are specifically expressed in the breast as a function of reproductive history.[9] Several of the parity-specific genes that we have initially isolated are markers of mammary epithelial cell differentiation, such as milk proteins. This finding suggests that the parous breast is more "differentiated" than the nulliparous breast and, as such, is consistent with the proposal made by Russo and Russo (2, 84) that parity protects against breast cancer by virtue of the differentiation that it induces. The developmental patterns of expression of milk protein genes are notably heterogeneous because each is up-regulated at a specific point in the alveolar differentiation pathway (85). Interestingly, we have found that the expression patterns of several of these genes reflect subtle aspects of reproductive history.[9] As such, studying the regulated expression of this class of genes as a function of reproductive history may provide insights into parity-related events in the breast. In addition, we have isolated a number of genes that are as yet unidentified. Given their interesting developmental patterns of regulation and parity-specific pattern of expression, these genes appear to represent an informative pool of candidate biomarkers for detecting changes in the breast associated with reproductive events.

In theory, the parity-specific pattern of expression for a given biomarker could reflect a global increase in expression of the gene in all mammary epithelial cells, an increase in the percentage of expressing cells in the breast, or both. We are analyzing the developmental pattern of expression of candidate genes by *in situ* hybridization to distinguish between these mechanisms. Our results indicate that parity-specific patterns of expression for different genes result from distinct developmental pathways. For example, these studies reveal examples of parity-dependent global changes in expression as well as parity-dependent changes in the abundance of expressing cells. This latter example is suggestive of a permanent pregnancy-induced expansion in the number of cells expressing a given biomarker in the breast.[9] These findings are consistent with the hypothesis that reproductive events may permanently alter the biology of the breast by differentially affecting subpopulations of cells.

We have also determined the impact of several reproductive parameters on the differential pattern of expression of these genes.[9] These experiments reveal that the parity-specific pattern of expression for some genes is independent of age, duration of postlactational regression, and age at first full-term pregnancy. In contrast, other genes we have identified are expressed in a parity-specific manner in the mammary glands of animals that have been mated as adolescents but not in the mammary glands of animals that have been mated as adults. These results suggest that the regulation of expression of such genes reflects developmental events in the mammary gland that are specific for age at first full-term pregnancy. These findings suggest that candidate cDNA biomarkers generated by these approaches may provide insight into subtle aspects of the molecular and cellular changes that occur in the breast as a result of parity. Ultimately, these studies are intended to gain sufficient understanding of the molecular pathways responsible for parity-induced protection against breast cancer in order to permit this naturally occurring protective event to be mimicked pharmacologically.

Summary

The current aims of this laboratory are designed to develop the molecular tools required to understand the relationship between nor-

[9] C. M. D'Cruz, J. Wang, S. I. Ha, and L. A. Chodosh, Reproductive history results in a permanent change in the expression of specific genes in the murine breast, manuscript in preparation.

mal mammary gland development and mammary carcinogenesis, as reflected in the epidemiology of reproductive endocrine risk factors for breast cancer. We have taken three approaches toward understanding this relationship, including: determining the role normally played by breast cancer susceptibility genes in mammary epithelial development; studying the function of three novel protein kinases in the breast; and identifying and analyzing genes that are specifically expressed in the breast during developmental stages associated with changes in breast cancer risk. We anticipate that these approaches will ultimately lead to a clearer understanding of the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

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Discussion

Dr. Andrew Feinberg: I have a really simple-minded question. These are very elegant studies, but I worry a bit about transgene-induced tumors, because in a sense you're starting with loaded dice. Aren't there any models of spontaneous mammary tumorigenesis? I thought there were some dogs or other species that developed cancer in a similar epidemiological manner as you mentioned for humans at the beginning. But, I don't know this field, so I may be totally wrong.

Dr. Chodosh: It is true that there are certain breeds of dogs that do develop breast cancer spontaneously, though I am not aware of any that show parity-induced protection against breast cancer. Obviously, how you choose a model to study a particular question is a central issue. Regarding transgenic rodent models of breast cancer there are a couple of points worth making.

The first is that our main experimental thrust is to look at the normal developmental biology of the breast. There is no question that the developmental stages through which the breast passes for both the mouse and the rat are exceedingly similar to what one finds in the human. That is, the developmental processes are as highly conserved as histology and tissue architecture.

The second is that it's quite clear from transgenesis experiments that many of the pathways that are altered during the process of carcinogenesis in the human breast cause similar problems in the rodent breast when altered by transgenic approaches. That is, the molecular pathways involved are highly conserved. So, while tumor development in a transgenic system is not "spontaneous" in the same way that we think of for human breast cancers, I would argue that the

history of cancer biology suggests that they are still quite useful models to examine pathways involved in development and carcinogenesis. So at the moment, as far as animals that we can work with, particularly those that we can genetically manipulate, we have mice. Similarly, in the rat, one is somewhat restricted to carcinogen-induced models, which may or may not faithfully mimic the processes involved in human carcinogenesis.

We think about the suitability of our model systems a great deal, and it's not clear to me that there's another *in vivo* system available at the present time that's more appropriate.

Speaker: Do you have any evidence these kinases play similar roles in the human breast? Because human breast cancer is quite different. Pathological studies are quite different from real breast cancer, because it's quite complicated by different pathways. So, my interest at the moment is that even if we are able to link these kinases to the set of human reactants, it is different with different types of breast cancer and different kinases being expressed. How do you plan to address these potential differences?

Dr. Chodosh: A very important question, which explains why we are moving into human tissue and human breast cancer cell lines to address some of these issues. This is information that we're currently gathering. The data that I showed you in human breast cancers and cancer cell lines are quite recent, so it's too preliminary for us to know whether there is some correlation between the expression of our kinases and Erb2 status or ER status, or a particular histological cell type. Regarding tumors that are marked by *Hunk* or *Punc* expression, clearly we would want to know whether they behave differently in terms of patient prognosis or response to therapy. We don't know that yet, though that's certainly something that we're very interested in.

Dr. Robert Ryan: I would like to ask, have you considered perhaps doing something like the chip-based assay where now you use the MMTV-neu and MMTV-c-myc breast cancer cell lines and test those samples for changes by looking at the various genes that are up-regulated or down-regulated. It might give you a handle on that, do you think?

Dr. Chodosh: Yes, that's certainly a possibility. In the context of DNA chip technology, I think we'd probably want to make the fewest possible changes that we could, starting with the most normal cells we can, then induce expression of a *Hunk* or *Punc* transgene and ask what genes are downstream, as opposed to using as a starting point tumor cell lines that obviously have undergone many unrelated changes over the long period of time they have been in culture. Certainly, I agree it's an important new technology.

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Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases

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Protein kinases frequently play key roles in the normal regulation of growth and development in eukaryotic organisms. As a consequence, aberrant expression or mutations in this family of molecules frequently result in transformation. Previously, we have conducted a screen to identify protein kinases that are expressed in the mouse during mammary gland development and in breast cancer cell lines. We now describe the molecular cloning, characterization and expression of Krct, a novel serine/threonine protein kinase unrelated to previously defined families of protein kinases. At the mRNA level, *Krct* is widely expressed throughout murine development and in adult tissues. Despite its ubiquitous expression, *Krct* is expressed preferentially within specific cellular compartments in multiple tissues, in particular within the testis and gastrointestinal tract. At the amino acid level, Krct is most closely related to four previously undescribed kinases in *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Caenorhabditis elegans*. Together, these kinases appear to define a novel subfamily of serine/threonine protein kinases. *Krct* possesses an unusually long 5′-untranslated region containing multiple upstream initiation codons and, in this regard, is similar to many proto-oncogenes that regulate normal growth and differentiation. In addition, *Krct* is located on mouse chromosome 11 closely linked to the epidermal growth factor receptor and, therefore, is likely to be co-amplified in a variety of human tumors.

INTRODUCTION

Epidemiologic evidence strongly suggests that breast cancer risk is intrinsically related to the manner in which the breast normally develops during fetal life, puberty and pregnancy (1–3). The close relationship between development and carcinogenesis in the mammary gland is exemplified by the biological properties of protein kinases. Studies in a variety of model systems have demonstrated that members of this family of regulatory molecules frequently modulate the normal growth, differentiation and development of eukaryotic organisms (4). As would be predicted from this, alterations in the regulated expression or function of protein kinases frequently have been found to result in cellular transformation and neoplasia (4–7). Indeed, several members of the protein kinase family have been shown to contribute to the development of breast cancer both in humans and in rodent model systems, including c-erbB-2/Neu, the epidermal growth factor (EGF) receptor, the insulin-like growth factor 1 (IGF1) receptor, the fibroblast growth factor (FGF) receptor family, Met and c-Src (8–12). Consistent with this, overexpression of an activated form of c-erbB2/Neu and of several other protein kinases in transgenic mice results in malignant transformation of the mammary epithelium. Moreover, some protein kinases have been shown to provide prognostic information relevant to clinical outcome and response to chemotherapy. In particular, amplification of c-*erbB2/Neu* in primary breast cancers has been reported to correlate with an aggressive tumor phenotype and a poor clinical prognosis (13–17).

To explore the role played by protein kinases in development and carcinogenesis in the mammary gland, we previously performed an RT–PCR-based screen to isolate cDNA fragments of protein kinases expressed during mammary gland development and in breast cancer cell lines. A total of 43 kinases were identified, three of which have not been described previously. One of the three cDNA fragments isolated encodes a portion of a novel protein kinase, Krct (kinase related to cerevisiae and thaliana), whose identification defines a novel subfamily of serine/threonine kinases within which Krct represents the first member to be identified in vertebrates. Here we describe the cloning, expression and initial characterization of murine Krct.

RESULTS

In order to identify molecules involved in regulating mammary gland development and carcinogenesis, we used a degenerate PCR approach to amplify catalytic subdomains of protein kinases expressed in breast cancer cell lines and in the mammary gland

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during its development. Poly(A)$^+$ RNA was prepared from murine mammary glands harvested at different developmental stages as well as from three non-transformed murine mammary epithelial cell lines and four breast cancer cell lines derived from different transgenic mouse models of breast cancer. First-strand cDNA from each of these sources was prepared independently and amplified using two sets of degenerate PCR primers encoding amino acid motifs within subdomains VIb and IX that are highly conserved among protein kinases (18–20). PCR products from each cDNA source were subcloned individually and screened by a combination of DNA sequencing and colony lift hybridization. Screening of ~1500 cDNA clones using this approach resulted in the identification of 43 protein kinases, including a single clone containing a 215 bp cDNA fragment, referred to as *Bstk2*, from the catalytic domain of a novel putative serine/threonine kinase (L.A. Chodosh *et al.*, in preparation). This cDNA fragment was isolated from amplified cDNA prepared from the mammary glands of mice that had undergone a single pregnancy followed by 21 days of lactation and 2 days of post-lactational regression.

Isolation of cDNA clones encoding Krct

In order to isolate a full-length cDNA clone encompassing the catalytic domain fragment *Bstk2*, this initial 215 bp RT–PCR product was used to screen cDNA libraries prepared either from murine mammary glands at day 7 of pregnancy, or from AC816, a mammary epithelial cell line derived from an adenocarcinoma arising in an MMTV–H-ras transgenic mouse. A total of 13 cDNA clones were obtained containing inserts ranging from 1.1 to 2 kb in size. The nucleotide sequence of clones M1-1 and M2A from the mammary gland cDNA library and clones D1 and G3 from the AC816 cDNA library was determined by automated sequencing of both strands. The composite 1512 nucleotide cDNA clone encoding Krct contains the entire 215 bp RT–PCR fragment *Bstk2* from position 743 to 957. This clone also contains an open reading frame (ORF) of 915 nucleotides beginning with an AUG at nucleotide 291 (Fig. 1). Comparison of the nucleotide sequence surrounding this putative initiation codon with the Kozak consensus sequence, GCC^A/$_G$CCAUGG, reveals matches at each of the most highly conserved positions: –6, –4, –3 and +4 (21,22). Multiple stop codons are present in all three frames upstream of the putative initiation codon. These data suggest that the initiation codon of *Krct* is the AUG at nucleotide 291. The 5′-untranslated region 5′-UTR of *Krct* is unusually long, relatively GC-rich (67%), and contains three upstream AUG codons, the closest of which is located 14 nucleotides upstream of the putative initiation codon. This upstream AUG initiates an ORF of 40 amino acids that overlaps the main *Krct* ORF. The 3′-UTR is 306 nucleotides in length and contains a polyadenylation signal and poly(A) tract. Additional cDNA clones were isolated containing small insertions and deletions within the coding region, the significance of which currently is under study.

The conceptual ORF of *Krct* comprises 305 amino acids and can be divided into an N-terminal domain of 19 amino acids, a 276 amino acid putative catalytic domain, and a 10 amino acid C-terminal domain. Each of the amino acids known to be invariant among protein kinases is present in the putative catalytic domain of Krct (23). Among the sequence elements that distinguish tyrosine kinases from serine/threonine kinases, Krct is most similar to the latter, in particular with regard to the LKPXN motif in subdomain VIb and the APE motif in subdomain VIII (Fig. 1). In addition, several amino acids in subdomains I, VII, VIII, X and XI that are conserved in tyrosine kinases are absent from the *Krct* ORF.

In order to confirm the coding potential of the *Krct*, *in vitro* transcription/translation (IVT) of the full-length cDNA clone was performed. This generated a predominant 35 kDa labeled polypeptide species, consistent with the predicted 34.4 kDa size of the protein (Fig. 2). Deletion of the entire 291 nucleotide 5′-UTR containing all three upstream AUGs resulted in the same 35 kDa labeled polypeptide. These observations strongly suggest that the predicted initiation codon at nucleotide 291 is indeed the site of translation initiation. Programing of reticulocyte lysates with the full-length *Krct* cDNA also generated two lesser species at 45 and 27 kDa. These species were also generated by IVT of a 5′-UTR-deleted form of *Krct*, demonstrating that these polypeptides do not result from translation initiation at upstream AUGs followed by readthough into the *Krct* ORF.

Homology to previously isolated protein kinases

Alignment of the predicted *Krct* ORF with GenBank sequences reveals that *Krct* displays highest homology to a small group of unpublished kinases found in lower eukaryotes. The two kinases demonstrating highest homology to *Krct* are atpk3 (GenBank accession no. U97568), a serine/threonine kinase of unknown function identified in *Arabidopsis thaliana*, and YPL236c (GenBank accession no. Z73592), a hypothetical protein recognized as a kinase following analysis of the complete *Saccharomyces cerevisiae* DNA sequence (24,25). Amino acid alignment of Krct to YPL236c and atpk3 reveals 35 and 34% overall identity to *Krct*, respectively, with significant homology over the length of the Krct N-terminal and kinase catalytic domains (Fig. 3A). In comparison, amino acid alignment of the catalytic domain of Krct to those of serine/threonine kinases from other kinase families in *S.cerevisiae* such as MekI and CaMKII reveals levels of identity on the order of 17–21%. YPL236c has no residues C-terminal to the catalytic domain, and atpk3 has only four, preventing analysis of similarities to Krct in this region. Krct also displays significant homology to a putative cyclin G-associated kinase identified in *A.thaliana* (GenBank accession no. AC003033), and to a putative serine/threonine protein kinase, D2045.7, identified in *Caenorhabditis elegans* (GenBank accession no. Z35639). Alignment of the C-terminus of the putative cyclin G-associated kinase to Krct reveals that four of the 10 C-terminal residues of Krct are conserved. Placement of Krct and its most closely related kinases into an evolutionary tree on the basis of amino acid similarities within their catalytic domains demonstrates that these kinases are more closely related to each other than to any previously described group of kinases (Fig. 3B). This suggests that Krct, YPL236c, atpk3, D2045.7 and AC003033 represent a novel subfamily of protein kinases, of which Krct is the first member identified in vertebrates.

Krct encodes a functional protein kinase

In order to demonstrate that *Krct* encodes a functional kinase, a fusion protein consisting of the entire coding sequence of *Krct* fused to glutathione-*S*-transferase (GST) was generated. *In vitro* kinase assays were performed with purified recombinant GST–Krct fusion protein using histone H1 and myelin basic protein (MBP) as substrates (Fig. 4). GST-fused Krct was able to phosphorylate both histone H1 and MBP *in vitro*. Autophospho-

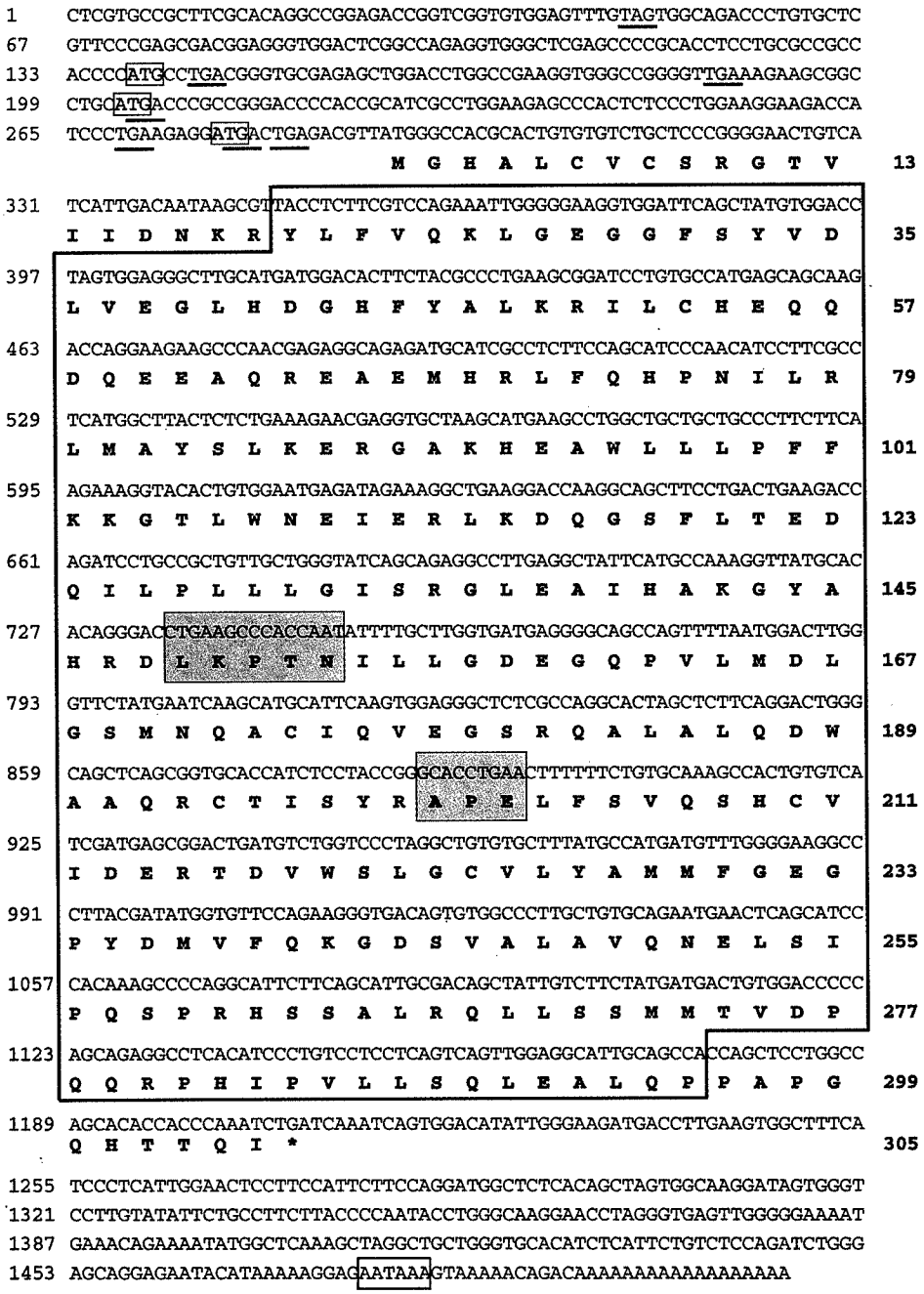

Figure 1. Nucleotide/amino acid sequence of *Krct*. The composite nucleic acid sequence and conceptual translation for *Krct* is shown. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in bold on the right. A light shaded box indicates the putative catalytic domain. Dark shaded boxes denote amino acid motifs characteristic of serine/threonine kinases. Upstream AUGs located in the 5′-UTR are indicated by shaded boxes. Stop codons present in the 5′-UTR and the polyadenylation sequence in the 3′-UTR are underlined. An asterisk denotes the stop codon for the *Krct* ORF.

rylation of the GST–Krct fusion protein was also observed. These findings confirm that *Krct* encodes a functional protein kinase.

Chromosomal localization

The mouse chromosomal localization of *Krct* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] mice (26). This interspecific backcross mapping panel has been typed for >2700 loci that are well distributed among all the autosomes as well as the X chromosome. C57BL/6J and *M.spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA *Krct* probe. The 5.0 kb *Hin*cII and 2.5 kb *Bam*HI *M.spretus* RFLPs (see Materials and Methods) were used to follow the segregation of the *Krct* locus in backcrossed mice. The mapping results indicated that

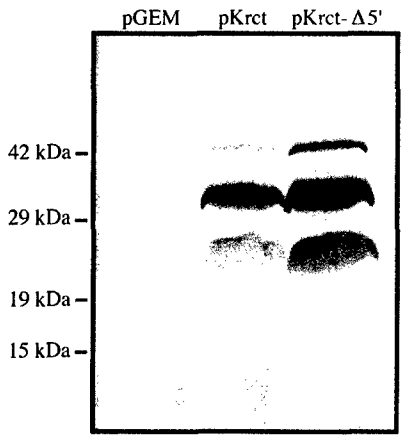

Figure 2. *In vitro* transcription/translation of *Krct*. [^{35}S]methionine-labeled Krct protein was generated using rabbit reticulocyte lysates programed with 500 ng of full-length *Krct* cDNA or 500 ng of p*Krct*-Δ5′, lacking the 291 nucleotide 5′-UTR. pGem11Zf plasmid was used as a control. The relative migration of molecular weight markers is indicated.

Krct is located in the proximal region of mouse chromosome 11 linked to *Ikaros*, *Egfr* and *Rel*. Although 125 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 174 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere–*Ikaros* (1/174)–*Egfr* (0/164)–*Krct* (6/133)–*Rel*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± standard error] are *Ikaros* (0.6 ± 0.6)–(*Egfr*, *Krct*) (4.5 ± 1.8). No recombinants were detected between *Egfr* and *Krct* in 164 animals typed in common, suggesting that the two loci are within 1.8 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 11 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided by the Mouse Genome Database, a computerized database maintained at the Jackson Laboratory, Bar Harbor, ME). *Krct* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown). The proximal region of mouse chromosome 11 shares homology with human chromosomes 7p and 2p (summar-

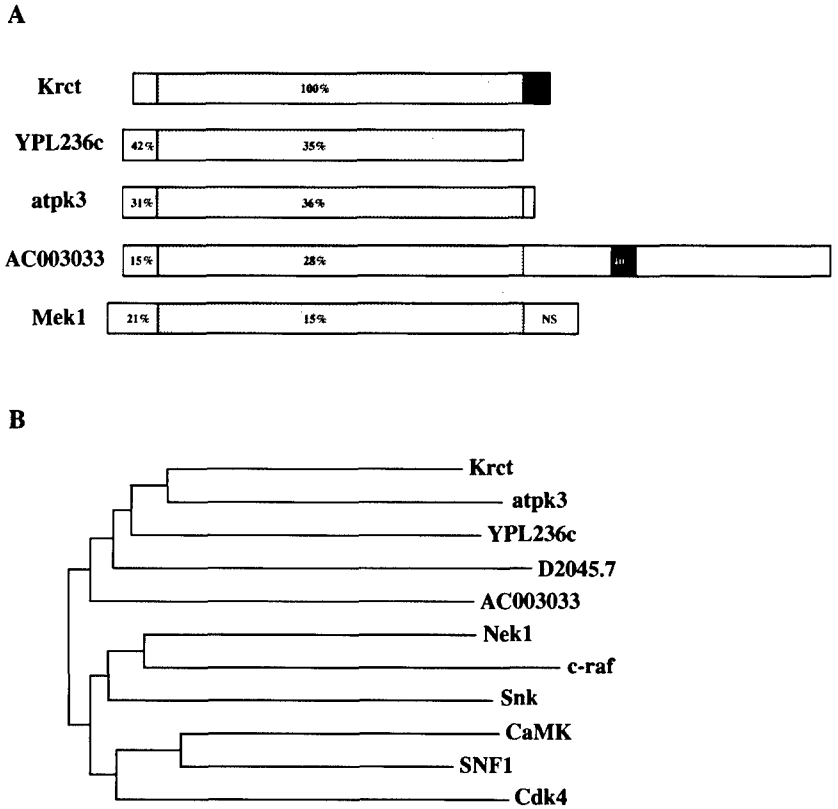

Figure 3. *Krct* defines a novel subfamily of serine/threonine kinases. (**A**) Schematic representation of amino acid homology between Krct, YPL236c, atpk3 and AC003033. Results of the pairwise analysis using the ClustalW alignment program of the N-terminus, catalytic domain and C-terminus of Krct, YPL236c, atpk3, AC003033 and Mek1 are listed. The percentage identity to Krct within each of these domains is indicated for each kinase. N-terminal, catalytic and C-terminal domains are indicated by open boxes, shaded boxes and black boxes, respectively. NS, not significant. (**B**) Phylogenetic tree illustrating the relationship of Krct with other serine/threonine kinases in the GenBank database. Analysis and depiction of results was performed using the ClustalX multisequence alignment program and DendroMaker 4.0.

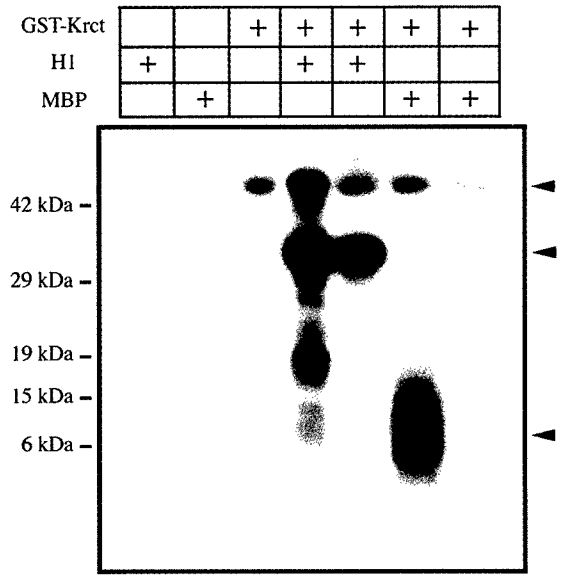

Figure 4. *Krct* encodes a functional protein kinase. Histone H1 and myelin basic protein (MBP) were used as *in vitro* kinase substrates for purified GST–Krct fusion protein. Histone H1 and MBP were incubated in the absence or presence of purified GST–Krct, as indicated. Reactions were performed using either 2 μg (lanes 3, 4 and 6) or 7 μg (lanes 5 and 7) of GST–Krct. Arrowheads indicate the relative migration of GST–Krct (top), histone H1 (middle) and MBP (bottom). The relative migration of molecular weight markers is indicated.

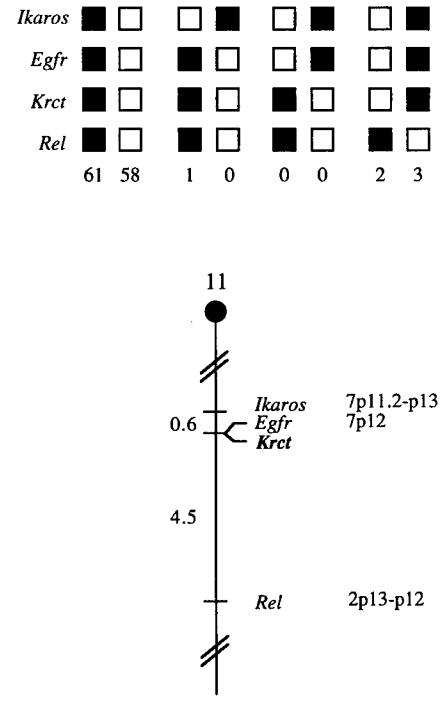

Figure 5. *Krct* maps in the proximal region of mouse chromosome 11. *Krct* was placed on mouse chromosome 11 by interspecific backcross analysis. The segregation patterns of *Krct* and flanking genes in 125 backcrossed animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, >125 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M.spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of an *M.spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 11 linkage map showing the location of *Krct* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in cM are shown to the left of the chromosome, and the positions of the loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the Genome Data Base, a computerized database of human linkage information maintained by The William H. Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

ized in Fig. 5). In particular, *Egfr* has been mapped to 7p12. The close linkage between *Egfr* and *Krct* in mouse suggests that the human homolog of *Krct* will map to 7p as well.

Analysis of *Krct* mRNA expression

In order to begin to analyze the biological role played by *Krct*, the spatial and temporal pattern of mRNA expression of this gene was determined during fetal development and in adult tissues in the mouse. Northern hybridization analysis of RNA isolated from FVB embryos at embryonic days E6.5, E13.5 and E18.5 using a *Krct* cDNA probe revealed an abundantly expressed 1.5 kb mRNA species at each of these developmental time points (Fig. 6A). The size of this message matches that predicted based on the size of the isolated cDNA clones, suggesting that the 1512 nucleotide *Krct* cDNA sequence represents the full-length message. *In situ* hybridization performed on E13.5 and E18.5 embryos using an antisense *Krct* cDNA probe revealed widespread *Krct* expression in most tissues of the developing mouse, with particularly high levels of expression observed in the fetal liver, dorsal root ganglia and cortical regions of the kidney at E13.5 and E18.5 (Fig. 6B). High levels of *Krct* expression were also observed in salivary gland, brown adipose tissue and hair follicles at E18.5.

Krct expression was also determined at the mRNA level in tissues of the adult mouse. Northern hybridization revealed that *Krct* is expressed in all organs examined, with highest levels of expression present in the mammary gland, ovary, liver, kidney and small intestine (Fig. 7). *In situ* hybridization analysis of *Krct* mRNA expression revealed that although *Krct* is widely expressed in tissues of the adult mouse, *Krct* expression within these tissues occurs preferentially in certain cell types. In the gastrointestinal tract, *Krct* is highly expressed in epithelial compartments of the stomach, duodenum and colon, and is expressed at significantly lower levels in the mesenchymal compartments of these tissues (Fig. 8). Similarly, *Krct* is expressed at higher levels in the glandular epithelium of the prostate than in its stromal component. In the mammary gland, *Krct* is expressed at high levels in the alveolar and ductal epithelium and at moderate though somewhat lower levels in the stroma. In the testis, *Krct* is expressed at high levels in the peripheral regions of seminiferous tubules that are populated principally by Sertoli cells, spermatogonia and primary spermatocytes. *Krct* is expressed at lower levels in Leydig cells within the interstitial compartment of the testis and in the central region of seminiferous tubules populated by the more differentiated spermatids and spermatozoa. All regions of the brain were found to express *Krct*, although expression was particularly high in the hippocampus and dentate gyrus (Fig. 9).

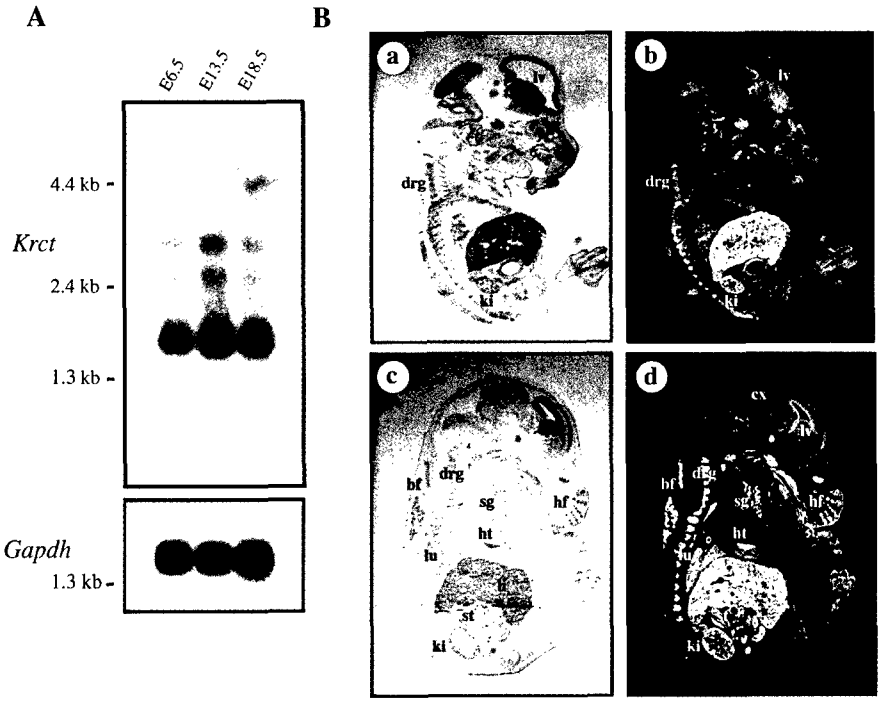

Figure 6. Expression of *Krct* during murine embryogenesis. (**A**) Northern hybridization analysis of 1 μg of poly(A)$^+$ RNA from day E6.5, E13.5 and E18.5 embryos hybridized with a cDNA probe specific for *Krct*. The blot was stripped and reprobed with *Gapdh* as a loading control. The relative migration of RNA size markers is indicated. (**B**) *In situ* hybridization analysis of *Krct* mRNA expression. Bright-field (a and c) and dark-field (b and d) photomicrographs of E13.5 (a and b) and E18.5 (c and d) FVB embryo sections hybridized with a *Krct* antisense cDNA probe. No signal over background was detected in serial sections hybridized with sense *Krct* probes. Magnification: 8× (a and b); 6.3× (c and d). bf, brown adipose tissue; cx, cortex; drg, dorsal root ganglia; hf, hair follicles; ht, heart; ki, kidney; li, liver; lu, lung; lv, lateral ventricle; sg, salivary gland; st, stomach.

DISCUSSION

The novel serine/threonine kinase, Krct, was isolated initially as a cDNA fragment in a degenerate oligonucleotide PCR screen designed to identify protein kinases potentially involved in mammary gland development and carcinogenesis. Herein we describe the molecular cloning and analysis of a full-length murine cDNA clone encoding *Krct*. *Krct* is widely expressed in the mouse at the mRNA level and encodes a 35 kDa protein with proven phosphotransferase capabilities. Amino acid sequence analysis indicates that Krct is most closely related to a group of unpublished kinases identified in *S.cerevisiae*, *A.thaliana* and *C.elegans* of unknown function. Comparison of the amino acid sequence of this group of genes with those from each of the major branches of the kinase superfamily suggests that these genes represent a novel subfamily of serine/threonine protein kinases, of which *Krct* represents the first member to be described in vertebrates.

Krct is expressed in the mouse in a broad range of tissues and at high levels throughout fetal development as well as in the adult mouse. In contrast to the widespread expression of *Krct* as determined by northern hybridization, *in situ* hybridization analysis reveals that *Krct* is expressed preferentially in specific cell types within these tissues. In particular, *Krct* is expressed preferentially in epithelial as compared with mesenchymal compartments of the prostate, stomach, duodenum, colon and, to a lesser extent, mammary gland. Similarly, *Krct* is expressed in the testis and brain in a cell type-specific pattern, being expressed at the highest levels in the testis in spermatogonia and primary spermatocytes, and in the brain in the hippocampus and dentate gyrus.

The 5′-UTR of *Krct* is 291 nucleotides in length. Interestingly, of 346 vertebrate mRNAs studied by Kozak, only 7.8% were longer than 200 nucleotides and only 2.3% were longer than 300 nucleotides (21). Long 5′-UTRs are generally associated with poor translational efficiency and/or translational regulation (21,22). Moreover, the 5′-UTR of *Krct* contains three upstream AUG codons, the most proximal of which lies only 14 nucleotides upstream of the initiation codon and initiates an ORF with an overlapping reading frame. Excluding proto-oncogenes, <10% of vertebrate mRNAs contain upstream AUGs, and the majority of those which do contain only one (21,22). In contrast, two-thirds of proto-oncogenes as well as a high percentage of growth factors, signal transduction proteins and transcription factors contain at least one upstream AUG. The unusual length of the 5′-UTR, as well as the presence and location of upstream initiation codons, suggest that *Krct* expression may be regulated at the level of translational initiation, and are consistent with a role for *Krct* in regulating normal growth and differentiation.

Our data indicate that *Krct* is located within 1.8 cM of the *Egfr* locus on mouse chromosome 11. *Egfr* is amplified and overexpressed in a variety of human tumors including glioblastomas and squamous cell carcinomas of the head and neck. Previous studies have demonstrated that additional genes located within 2 cM of *Egfr* are also amplified and overexpressed in glioblastomas (27). These data suggest that *Krct* may be contained on the *Egfr* amplicon and may be overexpressed in human cancers. Whether

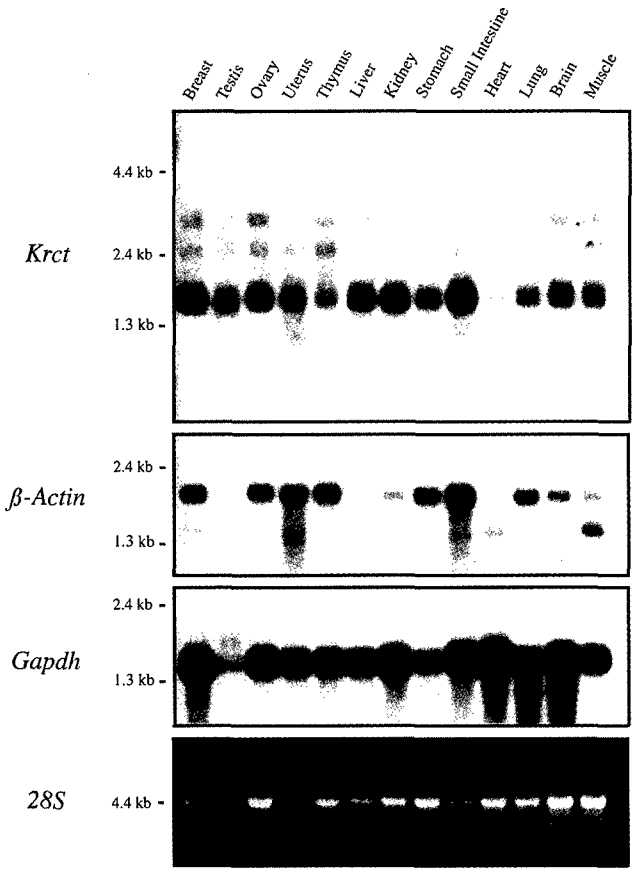

Figure 7. Northern hybridization analysis of *Krct* in tissues of the adult mouse. A northern blot containing 1 μg of poly(A)$^+$ RNA isolated from the indicated murine tissues was hybridized with a cDNA probe specific for *Krct*. The hybridization patterns for the same blot probed with a *b-actin* cDNA fragment, and a blot containing the same RNA samples probed with a *Gapdh* cDNA fragment, are shown. The 28S rRNA band is shown from the ethidium bromide-stained nitrocellulose blot.

such overexpression could contribute to the biological behavior of these tumors remains to be tested.

When considered with its cell type-specific pattern of expression, the cDNA structure and apparent conservation of this novel subfamily of protein kinases during evolution suggest that Krct may play a significant role in vital cellular processes. As such, it will be important to identify the signaling pathways in which Krct participates. In this regard, insights into these pathways may be gained through analysis of the functions of Krct family members in lower eukaryotes.

MATERIALS AND METHODS

Krct cloning

Poly(A)$^+$ RNA isolated from a H-*ras* transgenic mammary epithelial tumor cell line, AC816, and from the mammary glands of FVB mice at day 7 of pregnancy were each used to generate independent cDNA libraries in λZAP using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack II Gold packaging kit according to the manufacturer's instructions (Stratagene). A total of 5×10^5 plaques from each library were screened by standard methods using a [^{32}P]dCTP-labeled random-primed cDNA fragment (BMB Random Prime) corresponding to nucleotides 921–1135 of *Krct*. Hybridization was performed at a concentration of 10^6 c.p.m./ml in 48% formamide, 10% Dextran sulfate, 4.8× SSC, 20 mM Tris (pH 7.5), 10× Denhart's solution, 20 μg/ml salmon sperm DNA and 0.1% SDS at 42°C overnight. Following hybridization, washes were performed in 2× SSC/0.1% SDS. Filters were washed in 2× SSC/0.1% SDS at room temperature for 30 min (×2), followed by one 30 min wash at 50°C, and subjected to autoradiography (Kodak XAR-5). A total of 13 phage clones were plaque purified and plasmids liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). The nucleotide sequence of two clones from each library was determined by automated sequencing of both strands using an ABI Prism 377 DNA sequencer. The full-length *Krct* cDNA sequence has been deposited in the GenBank database (accession no. AF089869).

Tissue preparation

FVB mice were housed under barrier conditions with a 12 h light/dark cycle. Mouse embryos and mammary gland tissue were harvested at specified time points following timed matings. Day 0.5 p.c. was defined as noon of the day on which a vaginal plug was observed. Organs were harvested from 15- to 16-week-old virgin mice. Tissues used for RNA preparation were snap-frozen on dry ice. Tissues used for *in situ* hybridization analysis were either embedded in OCT medium and frozen in a dry ice/isopentane bath or fixed in neutral buffered formalin for 24 h followed by paraffin embedding.

Northern analysis

RNA was prepared by homogenization of cultured cells or snap-frozen tissue samples in guanidinium isothiocyanate followed by ultracentrifugation through cesium chloride as previously described (28,29). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia). Northern hybridization was performed as described (28). Probes used for analysis were generated by random-primed labeling of cDNA fragments and included nucleotides 624–1234 of *Krct*, 1142–1241 of *b-actin* and 466–1056 of *Gapdh*. Hybridization was performed overnight at 42°C. Following hybridization, washes were performed in 2× SSC/0.1% SDS at room temperature for 30 min (×2), followed by one 30 min wash at 50°C, and subjected to autoradiography (Kodak XAR-5).

In vitro transcription/translation

In vitro transcription/translation was performed on 500 ng of DNA using rabbit reticulocyte lysates and [^{35}S]methionine according to the manufacturer's instructions (Promega). Samples were electrophoresed on a 17% SDS–PAGE gel and subjected to autoradiography. *Krct* was subcloned into pGem11Zf (Promega) by *Eco*RI and *Apa*I digestion of the *Krct* D1 clone in pBS SK(+/–) and ligation of the purified *Krct* cDNA fragment (nucleotides 11–1512) into the *Eco*RI and *Apa*I sites of pGem11Zf. Deletion of the 5′-UTR was performed by PCR-based mutagenesis. Oligonucleotide primers CTGAATTCACTGAGACGTTATGGGCCACG, containing an *Eco*RI site, and CCCCTCATCACCAAGC were used to amplify nucleotides 280–767 of the D1 *Krct* cDNA clone using *Taq* polymerase (BMB). PCR was

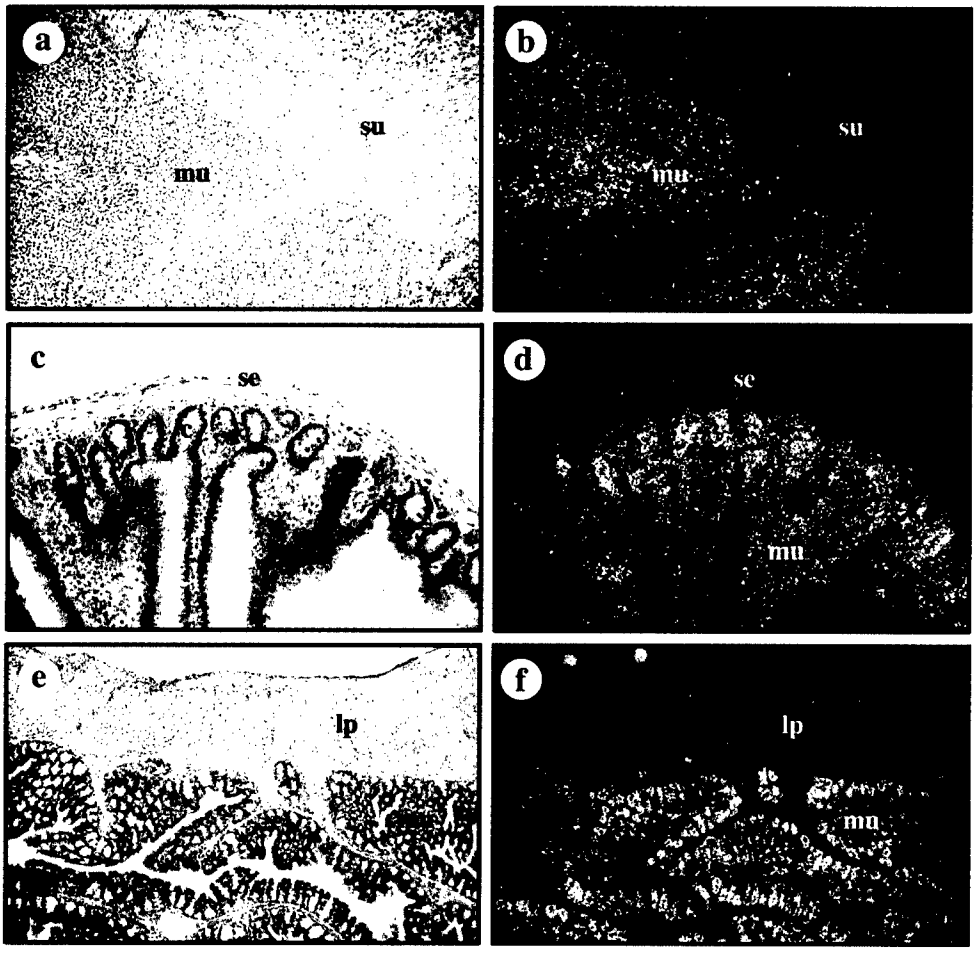

Figure 8. Spatial localization of *Krct* expression in the gastrointestinal tract. Bright-field (a, c and e) and dark-field (b, d and f) photomicrographs of *in situ* hybridization analysis performed on sections of stomach (**a** and **b**), duodenum (**c** and **d**) and colon (**e** and **f**) hybridized with a *Krct* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Krct* probe. Magnification: 100× (a and b); 200× (c–f). c, intestinal crypts; lp, lamina propria; mu, mucosa; se, serosa; su, submucosa.

performed in a Perkin Elmer GeneAmp PCR System 9600 thermocycler under the following conditions: initial denaturation at 95°C for 5 min followed by 30 s at 94°C, 30 s at 55°C and 2 min at 72°C for 10 cycles. A final extension was performed at 72°C for 5 min. The PCR product generated contained an *Eco*RI restriction site at the 5′ end and an internal *Bam*HI site at nucleotide 428 of *Krct*. This *Eco*RI–*Bam*HI fragment was purified and ligated into the D1 clone in pGem11Zf following digestion with *Eco*RI and *Bam*HI to yield a 5′-deleted form of *Krct*.

Kinase assay

The full-length *Krct* ORF was subcloned in-frame in pGEX6P-1 (Pharmacia) and expressed in bacterial strain BL21 by induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 3 h. Protein was purified by passage over glutathione–Sepharose 4B beads according to the manufacturer's instructions (Pharmacia). *In vitro* kinase activity was assayed under final reaction conditions consisting of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 100 μM ATP, using 3 μg of either histone H1 or MBP (Sigma) as a substrate, and either 2 or 7 μg of the purified bound GST–Krct fusion protein. Following a 30 min incubation at 30°C, samples were electrophoresed on a 15% SDS–PAGE gel and subjected to autoradiography.

In situ hybridization

In situ hybridization was performed as described (28). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using [^{35}S]UTP and [^{35}S]CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 624–1234 of *Krct*. Exposure times were: 4 weeks for OCT sections of colon, prostate, duodenum, brain and E18.5 embryo; 4 weeks 5 days for OCT sections of stomach, E13.5 embryo and mammary gland at day 7 of pregnancy; and 8 weeks 6 days for paraffin sections of testis.

Sequence analysis

Sequence analysis including predicted ORFs and calculation of the molecular weight of Krct was performed using MacVector. Pairwise sequence alignments were performed using the ClustalW alignment program. Multiple sequence alignment and

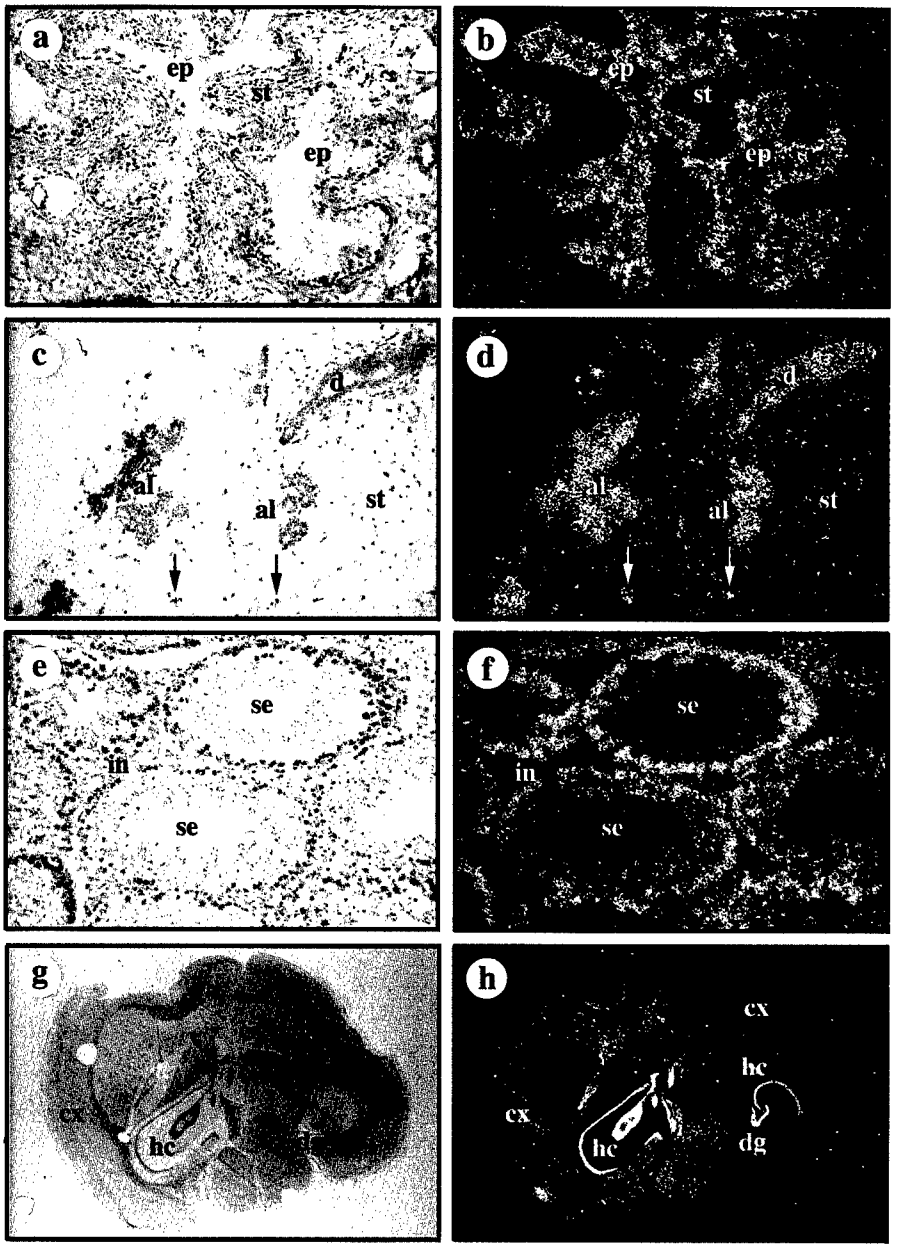

Figure 9. Spatial localization of *Krct* expression in tissues of the adult mouse. Bright-field (a, c, e and g) and dark-field (b, d, f and h) photomicrographs of *in situ* hybridization analysis performed on sections of dorsolateral prostate (**a** and **b**), number four mammary gland of a day 7 pregnant mouse (**c** and **d**), testis (**e** and **f**) and brain (**g** and **h**) hybridized with a *Krct* antisense probe. Arrows indicate *Krct*-expressing stromal cells in the mammary gland. No signal over background was detected in serial sections hybridized with a sense *Krct* probe. Magnification: 200× (a–f); 63× (g and h). al, alveoli; cx, cortex; d, duct; dg, dentate gyrus; ep; epithelium; hc, hippocampus; in; interstitium; st; stroma; se; seminiferous tubules.

phylogenetic calculations were performed using the ClustalX multisequence alignment program. DendroMaker 4.0 was used to draw a phylogenetic tree.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *M.spretus*)F_1 females and C57BL/6J males as described. In total, 205 N_2 mice were used to map the *Krct* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electropheresis, Southern blot transfer and hybridization were performed essentially as described. All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, an ~575 bp *Eco*RI fragment of mouse cDNA was labeled with [^{32}P]dCTP using a nick translation labeling kit (BMB); washing was carried out to a final stringency of 1× SSCP, 0.1% SDS, 65°C. Fragments of 6.0 and 3.6 kb were detected in *Hinc*II-digested C57BL/6J (B) DNA, and fragments of 5.0 and 3.6 kb were detected in *Hinc*II-digested *M.spretus* (S) DNA. In addition, *Bam*HI digestion produced fragments of 5.3 and 4.6 kb (B) and 5.3 and 2.5 kb (S). The presence or absence of the

M.spretus-specific fragments, which co-segregated, was followed in the backcrossed mice. The *Hin*cII and *Bam*HI data were combined.

A description of the probes and RFLPs for the loci linked to *Krct* including *Ikaros*, *Egfr* and *Rel* have been reported previously. Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events to explain the allele distribution patterns.

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NOTE ADDED IN PROOF

While this manuscript was under review, a kinase with nucleotide sequence nearly identical to that of Krct was described by J.M. Ligos, N. Gerwin, P. Fernandez, J.C. Gutierrez-Ramos and A. Bernad (1998) Cloning, expression analysis, and functional characterization of PKL12, a member of a new subfamily of ser/thr kinases. *Biochem. Biophys. Res. Commun.*, **249**, 380–384.

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Developmental Expression of *Brca2* Colocalizes with *Brca1* and Is Associated with Proliferation and Differentiation in Multiple Tissues

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Germline mutations in the putative tumor suppressor gene, *BRCA1,* predispose women to dramatically elevated risks of breast cancer, while germline mutations in the structurally unrelated gene, *BRCA2,* predispose both men and women to breast cancer. Recent studies have suggested an important developmental role for the murine homologue of *BRCA1* in the regulation of proliferation and differentiation. At the present time, however, little is known about the developmental role of *BRCA2* or the regulation of its expression *in vivo.* We have determined the spatial and temporal pattern of expression of the murine homologue of *BRCA2* during fetal development, in adult tissues, and in the mammary gland during postnatal development. Our results indicate that *Brca2* mRNA expression is highest in proliferating cellular compartments, particularly those undergoing differentiation. In the breast, *Brca2* expression is developmentally regulated and is induced during puberty and pregnancy and as a result of parity. Surprisingly, in multiple fetal and adult tissues the spatial and temporal pattern of *Brca2* mRNA expression is virtually indistinguishable from that of *Brca1,* despite the fact that these genes display no homology. These observations suggest that *Brca2* is involved in the processes of proliferation and differentiation in the mammary gland and other tissues, and that *Brca1* and *Brca2* mRNA expression may be regulated by similar pathways and stimuli in multiple cell types. Interestingly, however, our analysis reveals that *Brca1* and *Brca2* expression are differentially regulated during the development of specific endocrine target tissues, such as the testis during spermatogenesis and the breast during pregnancy. In addition, the ratio of mRNA expression in the mammary glands of adult females relative to adult males is significantly greater for *Brca1* than for *Brca2.* These observations imply that *Brca1* and *Brca2* mRNA expression are differentially regulated by sex hormones. In order to test this hypothesis, we have analyzed the expression of these two breast cancer susceptibility genes in ovariectomized mice treated with 17β-estradiol and progesterone. Our results demonstrate that the up-regulation of mRNA expression in the breast by ovarian hormones is significantly greater for *Brca1* than for *Brca2.* These observations suggest that the gender-specific differences in phenotype associated with germline mutations in *BRCA2* versus *BRCA1* may be related to the differential regulation of these genes by sex hormones. © 1997 Academic Press

INTRODUCTION

The loss of growth control characteristic of carcinogenesis is uniformly accompanied by alterations in normal pathways of differentiation and development. In the breast, the connection between carcinogenesis and development is illustrated by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy. The observation that the same reproductive endocrine events which control breast development also influence breast cancer risk supports the hypothesis that mammary gland development and mammary gland carcinogenesis are fundamentally related.

The epidemiological relationship between differentiation

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and carcinogenesis is illustrated on a molecular level by the existence and function of tumor suppressor genes. Germline mutations in these genes are associated with inherited cancer predisposition syndromes (Knudson, 1993). Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germline mutations in autosomal dominant susceptibility genes (Claus *et al.*, 1991; Newman *et al.*, 1988). In late 1994, one of these breast cancer susceptibility genes, *BRCA1*, was isolated by positional cloning (Hall *et al.*, 1990; Miki *et al.*, 1994). Germline mutations in this gene appear to account for most families with inherited breast and ovarian cancer and about half of families displaying inherited breast cancer alone (Easton *et al.*, 1993). Tumors arising in patients with germline *BRCA1* mutations typically display loss of the wild-type *BRCA1* allele, suggesting that *BRCA1* is a tumor suppressor gene (Smith *et al.*, 1992). Taken together with findings that reduction in *BRCA1* expression *in vitro* results in accelerated growth of breast and ovarian cancer cell lines, while overexpression of *BRCA1* results in inhibited growth of such cell lines, these observations are consistent with a model in which *BRCA1* negatively regulates proliferation in adult tissues (Holt *et al.*, 1996; Rao *et al.*, 1996; Thompson *et al.*, 1995). Interestingly, however, the murine homologue of *BRCA1* is expressed at highest levels in the mouse in cellular compartments containing rapidly proliferating cells, particularly those undergoing differentiation, such as in the breast during puberty and pregnancy (Lane *et al.*, 1995; Marquis *et al.*, 1995). The positive correlation between *Brca1* expression and cellular proliferation may be explained in part by the observation that the expression of this gene is regulated in a cell cycle-dependent fashion, with peak steady-state levels of mRNA and protein occurring at the G1/S transition (Chen *et al.*, 1996; Gudas *et al.*, 1996; Rajan *et al.*, 1996; Vaughn *et al.*, 1996b). These observations, together with the finding that *BRCA1* is a substrate for certain cyclin-dependent kinases, suggest a possible function for *BRCA1* in cell cycle checkpoint control and the regulation of proliferation (Chen *et al.*, 1996). Consistent with this hypothesis, several groups have now demonstrated that homozygous mutations in *Brca1* in mice result in abnormalities in cellular proliferation as well as early embryonic lethality (Gowen *et al.*, 1996; Hakem *et al.*, 1996; Liu *et al.*, 1996).

The existence of the breast cancer susceptibility gene, *BRCA2*, was initially suggested by the observation that the majority of breast cancer families with multiple cases of early-onset disease and at least one affected male are not linked to *BRCA1* (Stratton *et al.*, 1994). The *BRCA2* locus was subsequently mapped by linkage analysis to chromosome 13q12-13 and has recently been isolated by positional cloning (Tavtigian *et al.*, 1996; Wooster *et al.*, 1994, 1995). Like *BRCA1*, most women inheriting germline mutations in *BRCA2* have a high probability of developing breast cancer, although germline mutations in *BRCA2* appear to account for a smaller proportion of families with inherited breast cancer than those associated with *BRCA1* (Wooster *et al.*, 1994, 1995; Phelan *et al.*, 1996; Couch *et al.*, 1996b). In contrast to *BRCA1*, however, breast cancer incidence is markedly increased in men as well as in women inheriting mutations in *BRCA2*. Germline mutations in *BRCA2* also predispose female carriers to less severely elevated risks of ovarian cancer than those associated with *BRCA1* mutations (Couch *et al.*, 1996b; Phelan *et al.*, 1996; Tavtigian *et al.*, 1996; Thorlacius *et al.*, 1995, 1996; Wooster *et al.*, 1994, 1995). The supposition that *BRCA2* is a tumor suppressor gene is supported by the observation that most breast tumors arising in patients with germline *BRCA2* mutations exhibit LOH at the *BRCA2* locus, typically involving loss of the wild-type *BRCA2* allele (Collins *et al.*, 1995; Gudmundsson *et al.*, 1995). However, similar to the failure to find *BRCA1* mutations in sporadic breast cancers, only a small percentage of sporadic breast and ovarian tumors appear to carry somatic mutations in the *BRCA2* gene despite the fact that 25–30% of sporadic breast cancers show LOH at this locus (Lancaster *et al.*, 1996; Miki *et al.*, 1996; Teng *et al.*, 1996).

Breast cancer in men is a rare disease with an incidence approximately 100 times lower than that found in women (Crichlow, 1972; Meyskens *et al.*, 1976). Several risk factors for male breast cancer have an underlying endocrine component related to decreased ratios of androgens to estrogens (Meyskens *et al.*, 1976; Thomas *et al.*, 1992). This relationship is highlighted by the existence of families with inherited male breast cancer due to mutations in the androgen receptor (Wooster *et al.*, 1992). Since androgen response pathways are required for normal mammary gland development, and since epidemiological evidence suggests that alterations in these pathways predispose men to increased risks of mammary neoplasia, the potential regulation of *BRCA2* expression by steroid hormones is of considerable biological interest. That breast cancer incidence is not increased in men inheriting mutations in *BRCA1* suggests that the expression and/or function of *BRCA1* and *BRCA2* may differ in some tissues with respect to hormonal pathways involved in the regulation of growth and differentiation.

At the present time, little is known about *BRCA2* function or the regulation of its expression. *BRCA2* is a large gene from which an approximately 11-kb mRNA is transcribed (Couch *et al.*, 1996a; Tavtigian *et al.*, 1996; Wooster *et al.*, 1995). This transcript is predicted to encode a 3418-amino acid polypeptide showing little or no homology to previously identified proteins (Tavtigian *et al.*, 1996). Like *BRCA1*, few somatic mutations in *BRCA2* have been identified in sporadic breast tumors. Also like *BRCA1*, the numerous germline mutations that have been identified in *BRCA2* are distributed throughout the gene, and the majority of these mutations result in protein truncation (Couch *et al.*, 1996b; Neuhausen *et al.*, 1996; Phelan *et al.*, 1996; Tavtigian *et al.*, 1996; Thorlacius *et al.*, 1996; Wooster *et al.*, 1995).

Nevertheless, there are no apparent extended regions of nucleotide or amino acid homology between *BRCA1* and *BRCA2*. Recently, we and others have shown that *Brca2* mRNA expression *in vitro* is up-regulated in rapidly proliferating cells, is down-regulated in response to serum deprivation, and is expressed in a cell cycle-dependent manner peaking at the G1/S boundary (Rajan *et al.*, 1996; Vaughn *et al.*, 1996a). In addition, our studies indicate that *Brca2* expression is up-regulated in differentiating mammary epithelial cells independently of changes in proliferation. Interestingly, each of these changes in expression appears to occur coordinately with *Brca1* (Rajan *et al.*, 1996).

As a first step toward understanding the developmental and hormonal regulation of *Brca2*, we have analyzed the spatial and temporal pattern of expression of this gene in the mouse during fetal development, in adult tissues, in the mammary gland during postnatal development, and in ovariectomized animals treated with steroid hormones. We have found that *Brca2* is expressed at highest levels in proliferating cellular compartments, particularly those undergoing differentiation. Surprisingly, we have found that in multiple fetal and adult tissues the spatial and temporal pattern of *Brca2* mRNA expression is strikingly similar to that of *Brca1*, including the mammary gland during postnatal development. These observations imply that *Brca2* is involved in the processes of proliferation and differentiation in the mammary gland and other tissues, and that the expression of *Brca1* and *Brca2* may be regulated by similar pathways and stimuli in multiple cell types. Interestingly, we have found that the ratio of mRNA expression in the mammary glands of adult females relative to adult males is significantly greater for *Brca1* than for *Brca2*. In addition, we have shown that *Brca1* and *Brca2* expression are differentially regulated during the development of specific endocrine target tissues, such as the testis during spermatogenesis and the breast during pregnancy. In particular, our studies of ovariectomized mice treated with combinations 17β-estradiol and progesterone demonstrate that *Brca1* and *Brca2* mRNA expression in the breast are differentially regulated by sex hormones, raising the possibility that the elevated risk of breast cancer in men carrying germline mutations in *BRCA2* but not *BRCA1* may reflect this differential regulation.

MATERIALS AND METHODS

Animals and Tissues

FVB mice were used in all experiments and were housed under barrier conditions with a 12-hr light/dark cycle. Mouse embryos were harvested from timed natural matings of FVB mice. Day 0.5 p.c. was defined as noon of the day on which a vaginal plug was observed. Adult organs were harvested from 15- to 16-week-old female virgin mice. Tissues used for RNA preparation were snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT medium and frozen in a dry ice/isopentane bath. Mammary gland harvest consisted in all cases of the number three, four, and five mammary glands. The lymph node embedded in the number four mammary gland was removed prior to harvest. The mammary glands from between 10 and 40 age-matched mice were pooled for each developmental or experimental point.

For hormonal treatment experiments, adult female FVB mice were anesthetized and subject to bilateral oophorectomy. Two weeks later, mice began receiving daily subcutaneous injections with either phosphate-buffered saline (PBS) and gum arabic, 1 μg 17β-estradiol in PBS and gum arabic, 1 mg progesterone in gum arabic and PBS, or both 1 μg 17β-estradiol and 1 mg progesterone. Injections of either PBS or 17β-estradiol, and either gum arabic or progesterone, were given via separate injections each day for a total of 13–20 days, at which time mice were sacrificed for analysis.

RNA Preparation and Analysis

RNA was prepared by homogenization of snap-frozen tissue samples in guanidinium isothiocyanate with a polytron homogenizer, followed by ultracentrifugation through cesium chloride, as previously described (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia). Northern hybridization analysis was performed as described (Marquis *et al.*, 1995). Northern blots containing poly(A)$^+$ RNA derived from human tissues (Clontech) were processed according to the manufacturer's directions. DNA fragments used as probes included a 1.0-kb region of human *BRCA1* cDNA corresponding to amino acids 760 to 1099, the corresponding region of mouse *Brca1*, nucleotides 281 to 853 of the human *BRCA2* cDNA, or the corresponding region of the mouse *Brca2* cDNA (GenBank Accession No. U72947).

RNase protection analysis of *Brca1* mRNA expression was performed as described (Marquis *et al.*, 1995). RNase protection analysis of *Brca2* mRNA expression was performed as described for *Brca1* using a 240-bp fragment of mouse *Brca2* corresponding to amino acids 127 to 208 of human *BRCA2*. A 100-bp fragment of mouse β-actin corresponding to nucleotides 1142–1241 (Genbank Accession No. X03672) was subcloned into the pGEM-T vector (Promega) and processed as described above as an internal control along with either the *Brca2* or *Brca1* probes.

In Situ Hybridization

In situ hybridization was performed as described using a mouse *Brca2* cDNA corresponding to amino acids 19–208 of human *BRCA2*, a mouse *Brca1* cDNA corresponding to amino acids 727–1108 of murine *Brca1*, or nucleotides 542 to 768 of mouse $p21^{WAF1/CIP1}$ cDNA (Marquis *et al.*, 1995). Slides were developed at 10 days and 4 weeks and analyzed by bright- and dark-field microscopy using a Leica DMR microscope. Dark-field photomicrographs of sense controls were in all cases taken of sections adjacent to those used for the antisense photomicrograph. Shutter exposure times used in photographing sections hybridized to sense controls were in all cases equal to those used in photographing the antisense section.

RESULTS

mRNA Expression of the Murine Homologue of BRCA2

A cDNA fragment encoding the region of mouse *Brca2* corresponding to exons 2–6 of the human gene was isolated

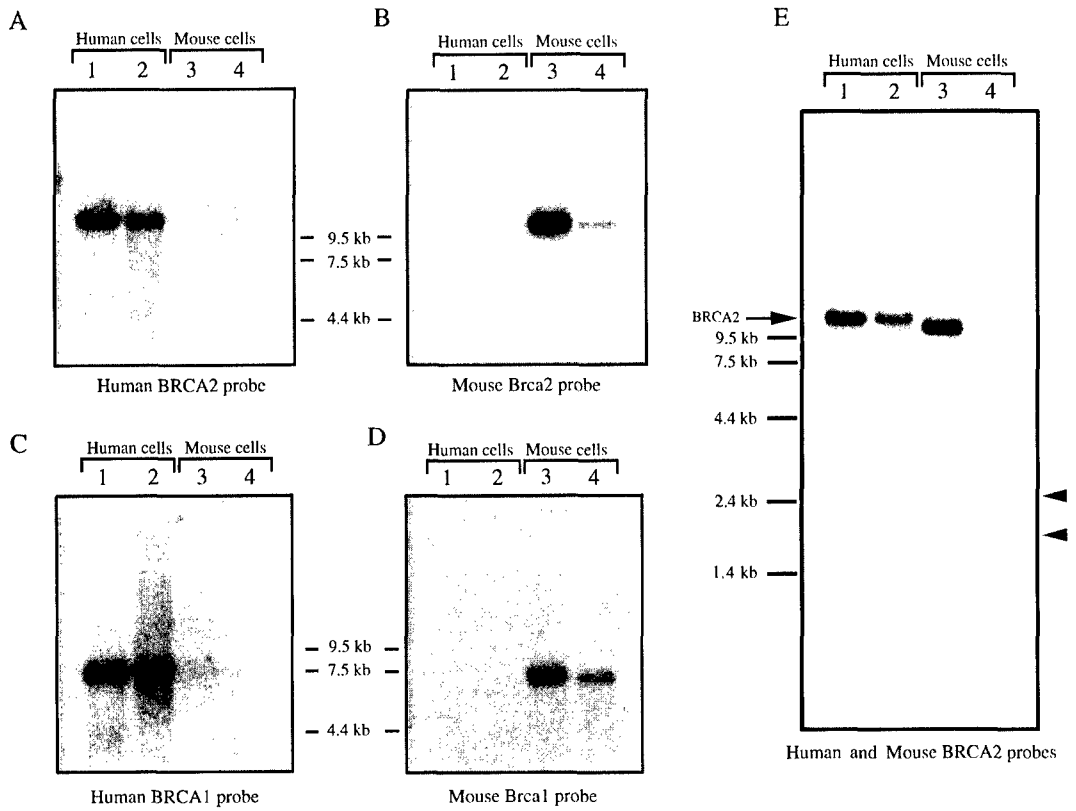
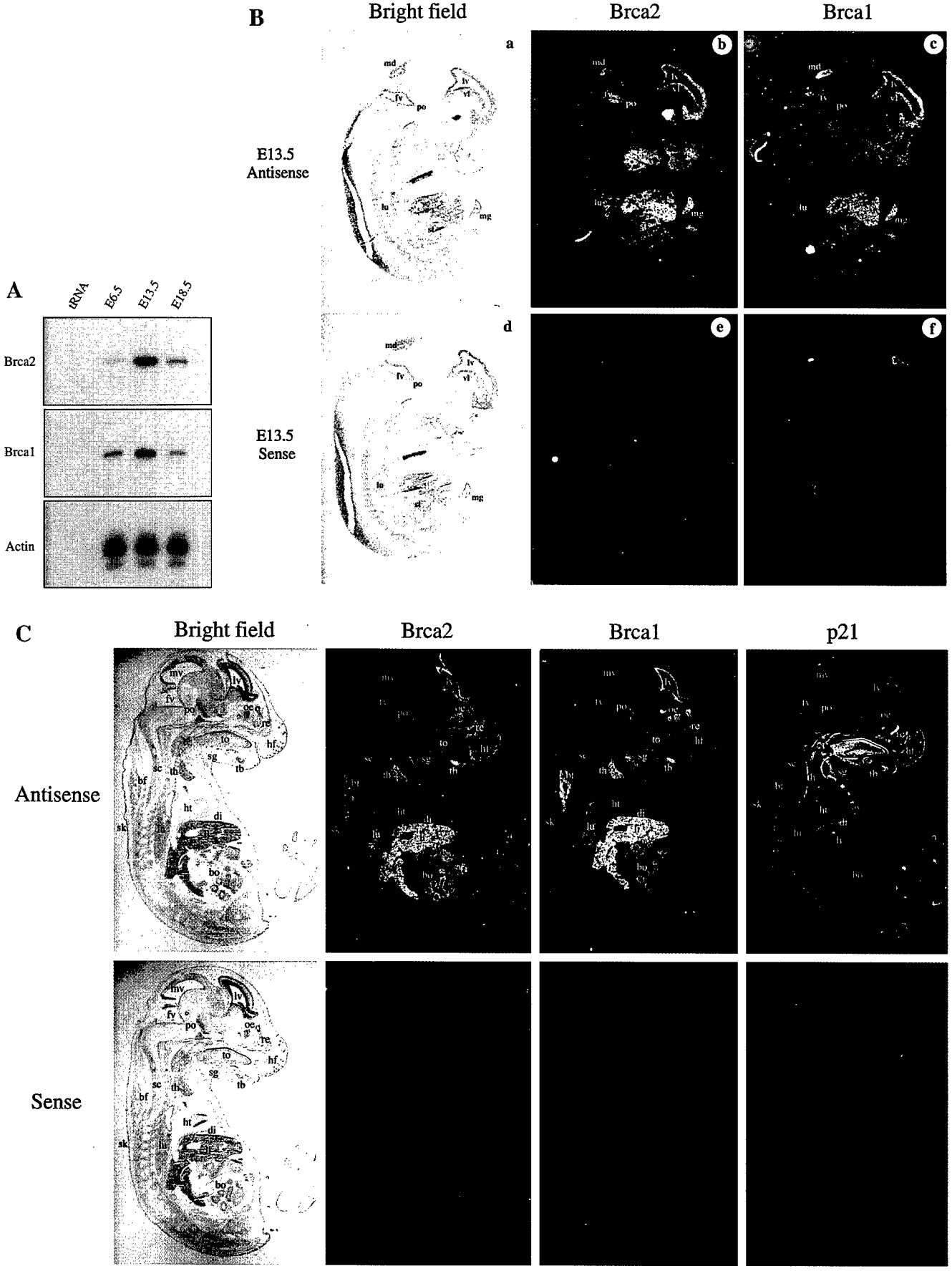


FIG. 1. Murine and human *BRCA2* mRNA transcripts are similar in size and do not cross-hybridize with *BRCA1*. Northern hybridization analysis of human *BRCA2* expression (A), mouse *Brca2* expression (B), human *BRCA1* expression (C), mouse *Brca1* expression (D), or human *BRCA2* and mouse *Brca2* expression (E) in human MCF-7 breast carcinoma cells (lane 1), human primary fibroblasts (lane 2), the nontransformed mouse mammary epithelial cell line HC11 (lane 3), or the *Ha-ras* overexpressing mouse breast cancer cell line, AC816 (lane 4). Northern blots contained 6 μg of poly(A)$^+$ RNA from each cell line. The relative migration of RNA molecular size standards is shown. The position of the major *BRCA2* transcript is indicated by an arrow. The positions of minor *BRCA2* transcripts are indicated by arrowheads.

by RT-PCR using murine mammary epithelial cDNA and primers directed against the 5′ end of human *BRCA2*. Comparison of the sequence of the resulting murine cDNA fragment, designated mBrca2.1, with *BRCA2* revealed 78% identity at the nucleic acid level and 80% similarity and 70% identity at the amino acid level. The sequence of mBrca2.1 was subsequently confirmed by comparison with overlapping cDNA and genomic clones. BLAST analysis of mBrca2.1 identified significant homology only to human *BRCA2*. Analysis of a murine genomic clone containing

FIG. 2. *Brca2* mRNA expression colocalizes with *Brca1* mRNA expression during murine embryonic development. (A) RNase protection analysis of *Brca2*, *Brca1*, and β-actin mRNA expression at Days 6.5, 13.5, and 18.5 of mouse embryonic development. 50 μg of total RNA from the indicated developmental time points was hybridized to ^{32}P-labeled antisense probes for *Brca2* or *Brca1*. A ^{32}P-labeled antisense probe for β-actin was included in each reaction as an internal control. (B) *In situ* hybridization analysis of *Brca2* and *Brca1* mRNA expression in Day 13.5 mouse embryos. Bright-field and dark-field photomicrographs of serial frozen sections from Day 13.5 mouse embryos hybridized to antisense (a–c) or sense (d–f) ^{35}S-labeled probes for *Brca2* (a, b, d, and e) or *Brca1* (c and f). (C) Bright-field and dark-field photomicrographs of serial frozen sections from sagitally sectioned Day 18.5 mouse embryos hybridized to ^{35}S-labeled antisense or sense probes for *Brca2*, *Brca1*, or *p21*$^{WAF1/CIP1}$, as indicated. bf, brown adipose tissue; bl, urinary bladder; bo, bowel; di, diaphragm; fv, fourth ventricle; hf, hair follicle; ht, heart; li, liver; lu, lung; lv, lateral ventricle of brain; mv, mesencephalic vesicle; md, roof of midbrain; mg, midgut; oe, olfactory epithelium; po, pons; re, respiratory epithelium; sc, spinal cord; sg, submandibular gland; sk, skin; st, stomach; tb, tooth bud; th, thymus; to, tongue; vl, ventricular layer of brain.


A
tRNA
E6.5
E13.5
E18.5
Brca2
Brca1
Actin
B
Bright field
Brca2
Brca1
E13.5
Antisense
E13.5
Sense
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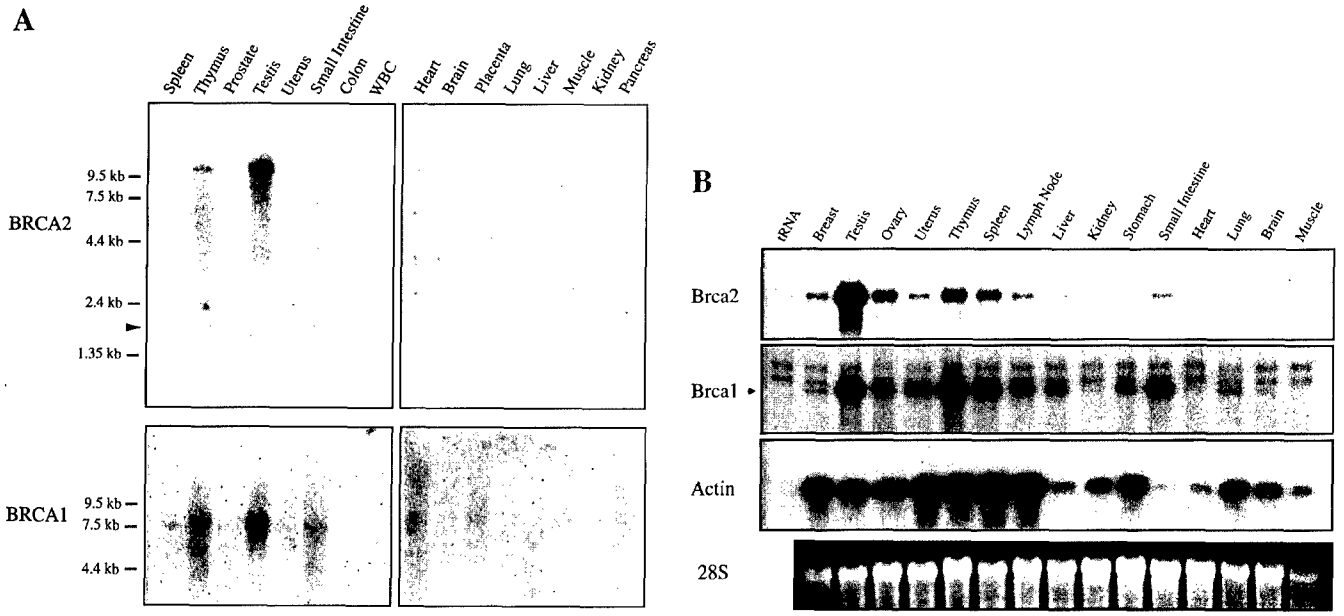

FIG. 3. Tissue-specific expression of human and mouse *Brca2*. (A) Expression of *BRCA2* and *BRCA1* mRNA in human tissues. Northern hybridization analysis of poly(A)$^+$ RNA isolated from the indicated human tissues (Clontech) with ^{32}P-labeled probes for either *BRCA2* or *BRCA1*. The relative migration of RNA molecular size markers is shown. The position of a minor *BRCA2* mRNA species is indicated by an arrowhead. (B) Expression of *Brca2* and *Brca1* mRNA in adult mouse organs. RNase protection analysis of mRNA isolated from the indicated tissues using ^{32}P-labeled antisense probes for *Brca2*, *Brca1*, or β-actin. A tRNA control shows the position of two nonspecific bands present in the *Brca1* probe. The position of the protected fragment corresponding to *Brca1* mRNA is indicated by an arrow. The 28S ribosomal RNA band is shown from an ethidium bromide-stained gel containing the same samples.

exons homologous to exons 2–10 of human *BRCA2* revealed the conservation of intron/exon boundaries (data not shown).

In order to address whether mBrca2.1 is derived from the bona fide murine homologue of *BRCA2* and to rule out the existence of cross-hybridization between *Brca1* and *Brca2* probes, identical Northern blots containing poly(A)$^+$ RNA prepared from both human and mouse cell lines were probed with radiolabeled cDNA fragments derived from corresponding regions of the cDNAs encoding human *BRCA2*, murine *Brca2*, human *BRCA1*, and murine *Brca1* (Fig. 1). A cDNA probe encoding exons 2–6 of human *BRCA2* detected a message approximately 11–11.5 kb in length in human but not in murine cell lines (Figs. 1A and 1E). Conversely, a murine probe derived from the corresponding region detected a message approximately 11 kb in length in murine, but not human, cells lines (Figs. 1B and 1E). Reprobing these blots with human *BRCA2* probes derived from either exon 11 or exons 20–24 yielded similar hybridization patterns (data not shown). A minor mRNA species 1.8 kb in length was detected in both murine and human samples, in addition to a 2.6-kb minor species detected in murine samples (Figs. 1E and 3A). An 11- to 12-kb *BRCA2* transcript has previously been detected in human testis and thymus (Couch *et al.*, 1996a; Tavtigian *et al.*, 1996). Exon 11-derived human and mouse *BRCA1* probes detected messages approximately 7.8 and 7.5 kb in length in human and mouse cell lines, respectively (Figs. 1C and 1D). No cross-hybridization was detected between murine *Brca2* and murine *Brca1* under any conditions. Weak cross-hybridization between murine Brca2.1 and human *BRCA2*, and between murine *Brca1* and human *BRCA1*, was detected only at lower washing stringencies (data not shown). Taken together, the sequence homology, genomic structure, mRNA size, and similar tissue distribution of expression (see below) strongly suggest that mBrca2.1 is derived from the bona fide murine homologue of *BRCA2*.

Brca2 Is Widely Expressed during Embryogenesis

The temporal pattern of *Brca2* mRNA expression during murine embryogenesis was examined by RNase protection analysis. Analysis of steady-state levels of *Brca2* mRNA during embryonic development revealed peak levels of *Brca2* mRNA expression at Day 13.5 of gestation and lower but detectable levels of expression at Days 6.5 and 18.5 (Fig. 2A). A similar pattern was observed for *Brca1* mRNA expression.

In order to determine the spatial localization of *Brca2* mRNA expression during fetal development, *in situ* hybridization was performed on frozen sections of Day 13.5 embryos using ^{35}S-labeled sense and antisense *Brca2* probes generated from the region corresponding to human exons 2–6. *Brca2* mRNA was widely expressed in Day 13.5 embryos with highest levels of expression observed in the fetal liver, midgut, the ventricular layer of the brain, and the germinal neuroblastic epithelium of the developing eye (Fig. 2B and data not shown). This pattern of expression was similar to that which we previously described for *Brca1* (Marquis *et al.*, 1995). *In situ* hybridization performed on adjacent sections of Day 13.5 embryos using antisense and sense probes for *Brca1* confirmed this similarity (Fig. 2B).

To extend these findings, *in situ* hybridization was performed on serial sections of Day 18.5 embryos using ^{35}S-labeled sense and antisense probes generated from *Brca2*, *Brca1*, or *p21*$^{WAF1/CIP1}$ (Fig. 2C). *p21*$^{WAF1/CIP1}$ was chosen as a control since it represents a gene known to be involved in the regulation of proliferation and differentiation in mammalian cells (Parker *et al.*, 1995). *In situ* hybridization analysis revealed intense organ-specific expression of *Brca2* mRNA in the liver, lung, bowel, salivary gland, thymus, tooth bud, brown adipose tissue, skin, olfactory epithelium, and ventricular layer of the brain. This pattern of expression suggests that *Brca2* is expressed during fetal development in cellular compartments characterized by rapid proliferation and differentiation. In addition, as observed in Day 13.5 embryos, each of the Day 18.5 fetal tissues displaying high levels of *Brca2* mRNA also exhibited high levels of *Brca1* mRNA. In fact, the spatial pattern and relative intensity of mRNA expression within each organ observed for *Brca2* and *Brca1* were virtually indistinguishable, with the exception of regions of the brain and spinal cord, which exhibited *Brca2* but not *Brca1* expression. In contrast, the pattern of expression observed for *Brca2* and *Brca1* differed markedly with that observed for *p21*$^{WAF1/CIP1}$ mRNA (Fig. 2C). *p21*$^{WAF1/CIP1}$ exhibited high levels of expression in skeletal muscle, cartilage, skin, nasal epithelium, and other terminally differentiated cells, consistent with prior observations (Parker *et al.*, 1995).

Brca2 Expression in Adult Tissues Reflects Patterns of Proliferation and Differentiation

The distribution of *Brca2* expression was analyzed in human and adult mouse tissues by Northern hybridization, RNase protection analysis, and *in situ* hybridization and was compared with that observed for *Brca1*. Like *Brca1*, the level of *Brca2* mRNA expression as detected by these methods was relatively low. Human *BRCA2* cDNA probes detected an mRNA species approximately 11 kb in length in human testis and thymus on Northern blots containing poly(A)$^+$ RNA derived from a variety of human tissues (Fig. 3A). Longer exposures of autoradiograms revealed detectable levels of expression in spleen, small intestine, and placenta (data not shown). Northern blots that were stripped and reprobed with a *BRCA1* cDNA probe derived from exon 11 revealed a tissue-specific pattern of mRNA expression for *BRCA1* similar to that for *BRCA2* (Fig. 3A).

Analysis of steady-state levels of *Brca2* mRNA in tissues of the adult mouse by RNase protection revealed a tissue-specific pattern of expression similar to that of human *BRCA2*, with high levels of expression observed in testis and moderate levels in thymus, spleen, and ovary (Fig. 3B). Lower but detectable levels of *Brca2* expression were observed in the breast and other tissues. RNase protection analysis of *Brca1* mRNA expression in these same samples revealed a pattern similar to that observed for *Brca2* (Fig. 3B).

The localization of *Brca2* mRNA expression in adult tissues, and its potential colocalization with *Brca1*, were investigated by *in situ* hybridization performed on serial tissue sections using probes for *Brca2*, *Brca1*, and *p21*$^{WAF1/CIP1}$ (Fig. 4A). *Brca2* mRNA expression in the breast was principally restricted to the epithelium (Figs. 4A and 6D). *Brca2* expression in the ovary was predominantly follicular, with the highest levels of mRNA found in granulosa cells in developing follicles and relatively lower levels in stromal cells. *Brca2* mRNA expression in the duodenum was found predominantly in the basal epithelial cell layer located at the base of the intestinal crypts, areas known to contain rapidly dividing cell types undergoing differentiation. In the uterus, *Brca2* expression was observed at highest levels in the glandular portions of the endometrium with moderate levels of expression observed throughout the stroma. Strikingly, as observed during fetal development, the spatial pattern of *Brca2* mRNA expression in the breast, ovary, duodenum, and uterus was virtually identical to that observed for *Brca1*. This pattern differed markedly from that observed for *p21*$^{WAF1/CIP1}$, whose expression occurred at highest levels in terminally differentiated cell types such as the columnar cells of the gastric and intestinal epithelium found most distal to the crypts (Fig. 4A and Parker *et al.*, 1995).

The spatial patterns of *Brca2* mRNA expression in the testis, thymus, prostate, liver, and spleen were also similar to those previously described for *Brca1* (Fig. 4B). In addition, *in situ* hybridization analysis confirmed the presence of *Brca2*, but not *Brca1*, mRNA expression in the brain of adult animals, consistent with results obtained from RNase protection analysis (Fig. 3B and 4B). An additional difference between *Brca1* and *Brca2* expression in adult tissues was revealed by *in situ* hybridization studies of the stomach. While both *Brca1* and *Brca2* expression in the stomach were found principally in the basal epithelial cell layer at the base of the mucosal epithelium, significant expression of *Brca2*, but not *Brca1*, was also observed in the more differentiated epithelial cells migrating towards the tips of the gastric villi (Fig. 4A). Thus, as observed during fetal development, *Brca2* mRNA expression in the adult was found in a wide variety of tissues, was generally associated with proliferating cellular compartments undergoing differentiation, and was strikingly similar to that observed for *Brca1*. De-

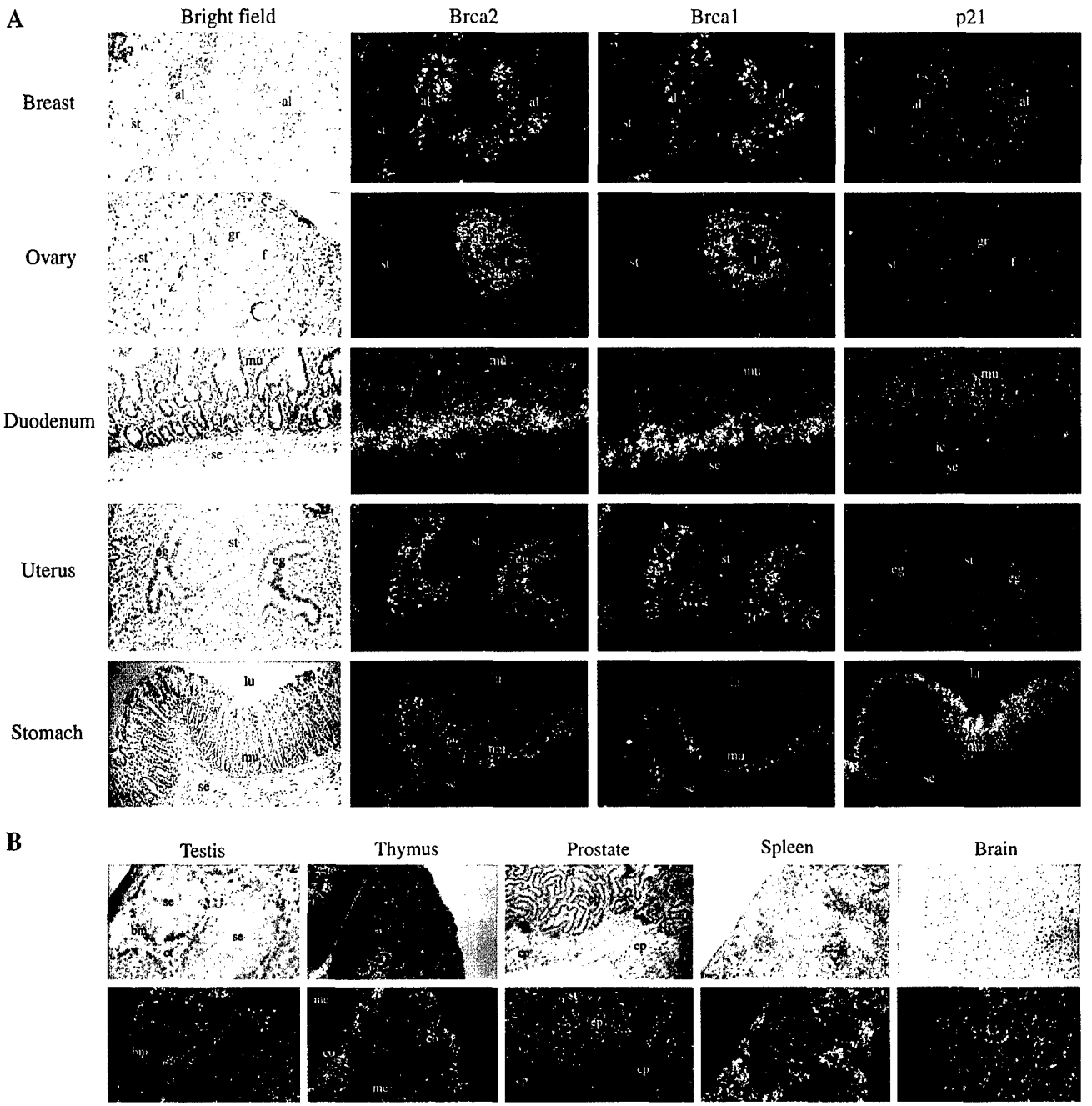

FIG. 4. *Brca2* is expressed in proliferating cellular compartments in adult tissues (A) *Brca2* mRNA expression colocalizes with *Brca1* mRNA expression in adult mouse organs. Bright-field and dark-field (antisense) photomicrographs of *in situ* hybridization analysis performed on serial frozen sections of the indicated tissues using ^{35}S-labeled antisense probes for *Brca2*, *Brca1*, or *p21*$^{WAF1/CIP1}$, as indicated. No signal was detected over background in serial sections hybridized with sense *Brca2*, *Brca1*, or *p21*$^{WAF1/CIP1}$ probes (data not shown). al, developing alveoli in mammary gland of pregnant animal; st, stroma; f, developing follicle; gr, granulosa cells; mu, intestinal or gastric mucosa; ic, intestinal crypts; se, serosa; eg, endometrial glands; lu, lumen of bowel. (B) Localization of *Brca2* mRNA expression in additional tissues of the adult mouse. Bright field and dark field (antisense) photomicrographs of *in situ* hybridization analysis performed on frozen sections of the indicated tissues using an ^{35}S-labeled antisense probe for *Brca2*. No signal was detected over background in serial sections hybridized with a sense *Brca2* probe (data not shown). bm, basement membrane; co, thymic cortex; cp, capsule; ct, connective tissue; ep, epithelium; me, thymic medulla; se, seminferous tubule; st, stroma.

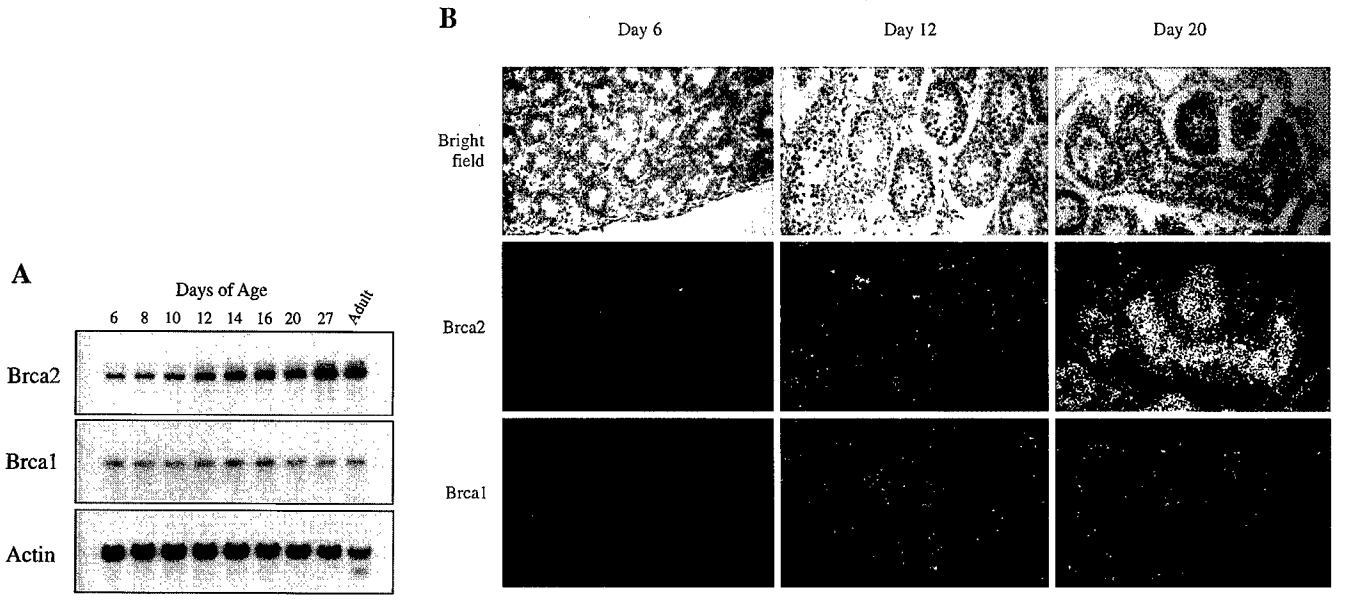

FIG. 5. Developmental up-regulation of *Brca2*, but not *Brca1*, mRNA expression during spermatogenesis. (A) RNase protection analysis of *Brca2* and *Brca1* developmental expression during spermatogenesis. RNase protection analysis of total RNA isolated from the testes of mice at the indicated ages using antisense probes for *Brca2*, *Brca1*, or β-actin. (B) *In situ* hybridization analysis of *Brca2* and *Brca1* developmental expression during spermatogenesis. Bright-field and dark-field photomicrographs of *in situ* hybridization analysis performed on serial sections of testis harvested from mice at the indicated ages using antisense probes for *Brca2* or *Brca1*. To facilitate comparison, all dark-field photomicrographs shown were taken using identical shutter exposure times.

spite this similarity, however, the spatial patterns of *Brca2* and *Brca1* expression were distinguishable in some tissues.

Brca2 Expression, but Not Brca1 Expression, Is Up-Regulated during Spermatogenesis

The high levels of *Brca2* expression observed in the testis, the localization of *Brca2* expression in cellular compartments involved in proliferation and differentiation, and the gender-specific aspects of the phenotype resulting from germline *BRCA2* mutations compared with germline mutations in *BRCA1* prompted us to examine *Brca2* mRNA expression during spermatogenesis. The analysis of differentiation in this system is facilitated by the fact that while in adult mice the various differentiating cell types appear simultaneously, during the first round of spermatogenesis which occurs during the first few weeks of life cell types characteristic of each of the stages of differentiation appear synchronously and sequentially as enriched populations (Janca *et al.*, 1986; Nebel *et al.*, 1961). Steady-state levels of *Brca2* mRNA in the testis were analyzed by RNase protection as a function of age from 6 to 27 days following birth (Fig. 5A). This analysis revealed that steady-state levels of *Brca2* mRNA progressively rise in the developing testis beginning at about Day 12 of life, peaking by approximately 4 weeks of age, and remaining high in adult life. In contrast, steady-state levels of *Brca1* mRNA were essentially constant during this same period of postnatal development. *In situ* hybridization analysis of testes harvested from mice during the first 3 weeks of life confirmed the up-regulation of *Brca2* mRNA as well as the relatively constant level of expression of *Brca1* mRNA and further suggested that this up-regulation was global in nature, resulting from increased steady-state levels of *Brca2* mRNA in the majority of cells in the seminiferous tubule rather than from an increase in the fraction of expressing cells (Fig. 5B). These results support the hypothesis that *Brca2* plays a role in the regulation of proliferation and/or differentiation during spermatogenesis. The differences observed in developmental expression patterns between *Brca1* and *Brca2* during this process may reflect underlying differences in the patterns of regulation or functions of these genes, particularly those relevant to androgen-responsive tissues.

Brca2 Expression Is Induced in the Mammary Gland during Puberty and Pregnancy

RNase protection analysis and *in situ* hybridization were used to determine the spatial and temporal pattern of *Brca2* expression during the postnatal development of the mammary gland (Fig. 6). Throughout the course of mammary gland development, *Brca2* mRNA was expressed predominantly in epi-

thelial cells, with lower but detectable levels of expression observed in the stromal portion of the gland (Figs. 4A and 6D). This observation is consistent with the fact that germline mutations in *BRCA2* predispose carriers to malignant transformation of the mammary epithelium. *Brca2* mRNA levels in the mammary glands of 2- and 5-week-old immature virgin females were significantly higher than those found in the mammary glands of 10- and 15-week-old mature virgin females (Figs. 6A and 6E). This is clearly evident upon normalization of steady-state levels of *Brca2* mRNA to those of β-actin (Fig. 6B), which is required to distinguish genuine regulatory effects from nonspecific dilutional effects resulting from the large-scale accumulation of milk protein mRNAs during late pregnancy and lactation (Buhler *et al.*, 1993; Gavin and McMahon, 1992; Marquis *et al.*, 1995). Consistent with the higher steady-state levels of *Brca2* mRNA observed in the mammary glands of immature mice by RNase protection, *in situ* hybridization performed on the mammary glands of immature mice revealed higher levels of *Brca2* mRNA in terminal end buds than in adjacent ducts (Fig. 6E and data not shown). Terminal end buds are undifferentiated structures which are present in the breast primarily during puberty and which contain rapidly proliferating cell types undergoing differentiation. *In situ* hybridization performed on the mammary glands of adult virgin mice confirmed the low levels of *Brca2* mRNA present in the mammary epithelium of mature mice (Fig. 6E). The decrease in *Brca2* mRNA levels observed following puberty is consistent with the elevated level of *Brca2* expression found in terminal end buds relative to mature ducts and parallels the disappearance of these structures at the completion of ductal morphogenesis. As assayed by RNase protection, *Brca2* mRNA levels in the female breast increased sharply early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (Figs. 6A and 6B). *In situ* hybridization demonstrated that this up-regulation of *Brca2* expression occurred preferentially in developing alveoli as compared with adjacent epithelial ducts (Fig. 6E). *Brca2* expression declined during the remainder of pregnancy (Days 14 and 20), reaching its nadir during lactation and early postlactational regression as assayed by RNase protection and *in situ* hybridization (Figs. 6A, 6B, and 6E). Interestingly, RNase protection analysis revealed that the mammary glands of parous animals which had undergone four weeks of postlactational regression expressed higher levels of *Brca2* mRNA than the mammary glands of age-matched virgin controls (Figs. 6A and 6B).

The parallel determination of steady-state levels of *Brca1* mRNA in the same samples used to analyze *Brca2* expression during mammary gland development revealed several interesting points (Figs. 6A and 6C). Like *Brca2*, *Brca1* is predominantly expressed in the mammary epithelium throughout the postnatal development of this organ (Lane *et al.*, 1995; Marquis *et al.*, 1995). Also like *Brca2*, *Brca1* mRNA levels are up-regulated during puberty, pregnancy, and as a result of parity (Figs. 6A and 6C and Marquis *et al.*, 1995). Moreover, both *Brca1* and *Brca2* are expressed at significantly higher levels in the female breast compared to the male breast, presumably reflecting the greater amount of mammary epithelium present in the adult female (Fig. 6D). However, two intriguing differences were noted in the quantitative patterns of expression of these two genes during mammary gland development. First the magnitude of the up-regulation in *Brca1* mRNA levels that occurred in the breast during early pregnancy was approximately twice that observed for *Brca2* (Figs. 6B and 6C). Second, quantitative analysis of steady-state *Brca2* and *Brca1* mRNA levels in multiple independent pooled mammary gland samples corresponding to over 200 age-matched male and female mice revealed that the ratio of mRNA expression in the mammary glands of adult females relative to adult males was significantly greater for *Brca1* than for *Brca2* (Fig. 6D). These observations suggest that androgens and/or ovarian hormones differentially regulate the expression of *Brca1* and *Brca2* at the mRNA level.

Brca2 and Brca1 Expression Are Differentially Regulated by Ovarian Hormones

The observation that *Brca2* mRNA levels are up-regulated during pregnancy suggested that the expression of this gene may be modulated directly or indirectly by ovarian hormones. Moreover, the observation that the up-regulation of *Brca2* mRNA levels during early pregnancy is less marked than that observed for *Brca1*, and that the ratio of mRNA expression in the mammary glands of female relative to male mice is significantly greater for *Brca1* than for *Brca2*, suggested that the expression of these two genes may respond to ovarian hormones in a quantitatively different manner. In order to test this hypothesis *in vivo*, the effect of ovariectomy on *Brca1* and *Brca2* mRNA expression levels was determined. Steady-state levels of *Brca1* and *Brca2* mRNA were significantly lower in the mammary glands of ovariectomized mice compared with age-matched intact animals (Fig. 7A). That this decrease was due at least in part to the loss of ovarian hormones was suggested by the observation that steady-state levels of *Brca2* mRNA were restored in ovariectomized mice by treatment with a combination of 17β-estradiol and progesterone. By comparison, the induction of *Brca1* mRNA expression by estradiol and progesterone was significantly greater than that observed for *Brca2* (Fig. 7A). The relative induction of *Brca1* and *Brca2* expression by each of these hormones was investigated in ovariectomized mice (Fig. 7B). Consistent with our previous observations, as assayed by RNase protection *Brca1* mRNA levels were low in ovariectomized animals and increased slightly in response to 17β-estradiol alone, moderately in response to progesterone alone, and synergistically in response to a combination of these two hormones (Fig. 7B and Marquis *et al.*, 1995). By comparison, the induction of *Brca2* expression in the mammary glands of animals treated with a combination of 17β-estradiol and

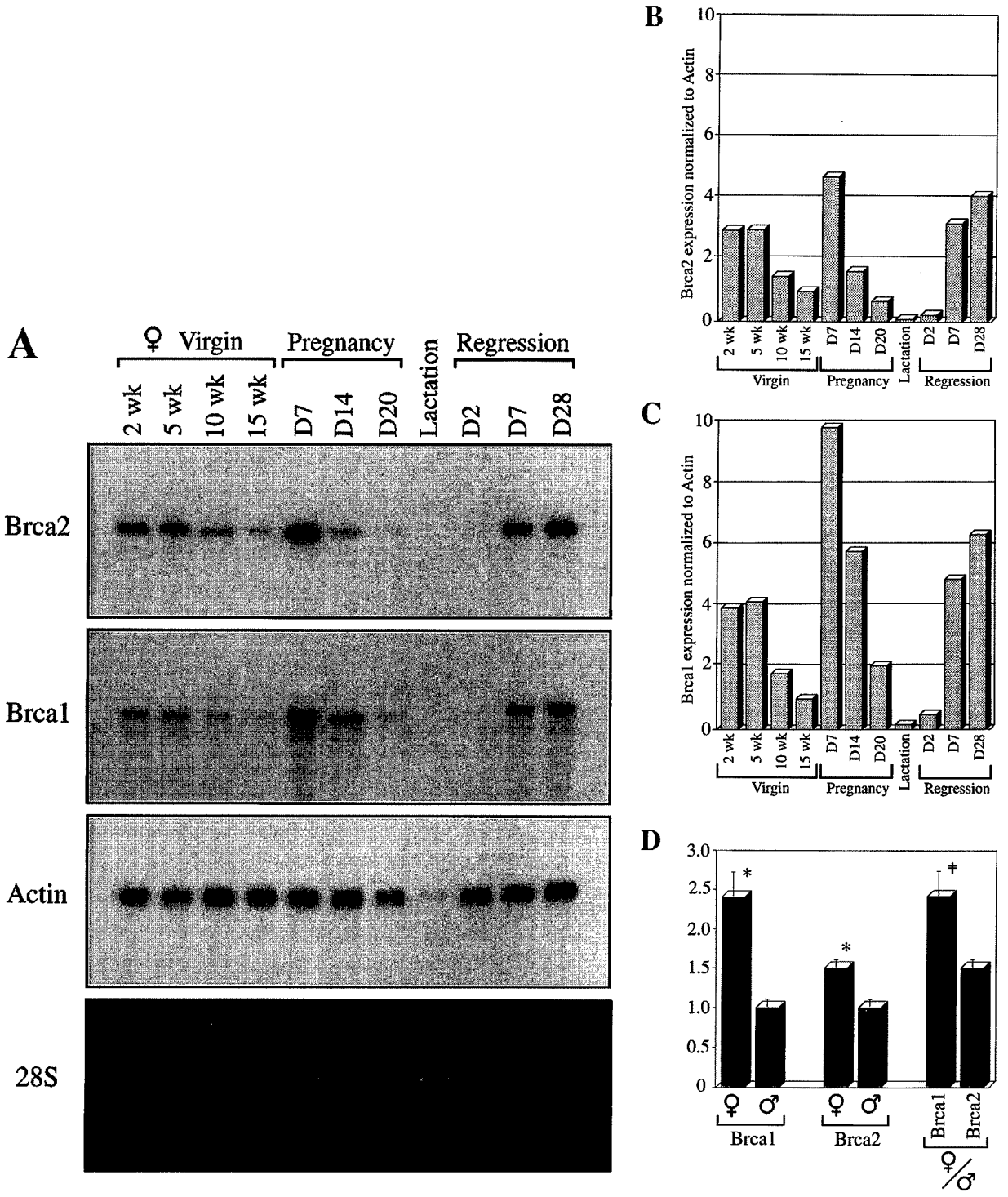

FIG. 6. Coordinate expression of *Brca2* and *Brca1* during postnatal mammary gland development. (A) RNase protection analysis of mRNA isolated from the mammary glands of mice at the indicated developmental stages using ^{32}P-labeled antisense probes for *Brca2*, *Brca1*, or β-actin. The 28S ribosomal RNA band is shown from an ethidium bromide-stained gel containing the same samples. (B) Phosphorimager quantitation of *Brca2* mRNA expression shown in (A). *Brca2* mRNA expression is normalized to β-actin mRNA expression to correct for dilutional changes in signal intensity resulting from large-scale increases in milk protein mRNA production during late pregnancy and lactation. The *Brca2*/actin ratio is defined as 1.0 for the 15-week virgin sample. (C) Phosphorimager quantitation of *Brca1*

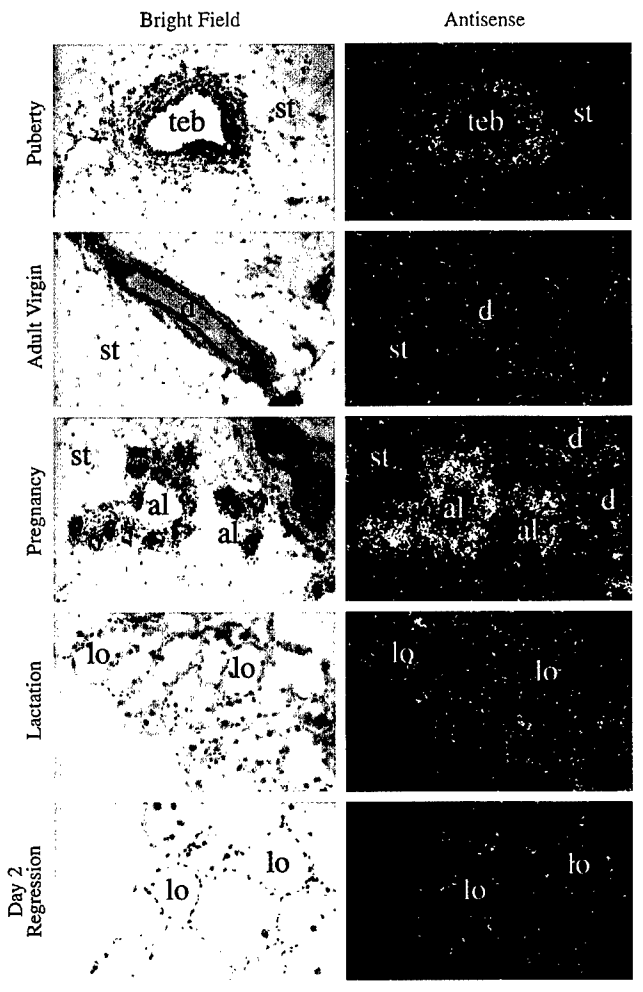

FIG. 6—*Continued*

progesterone was significantly less marked than that for *Brca1* (Fig. 7B).

These observations were confirmed by *in situ* hybridization performed on mammary glands harvested from the same experimental animals (Fig. 7C). *Brca2* and *Brca1* mRNA levels both declined in the mammary epithelium of ovariectomized relative to sham-operated animals. Treatment of ovariectomized animals with both 17β-estradiol and progesterone resulted in the synergistic up-regulation of *Brca1* mRNA expression. In contrast, consistent with our RNase protection results, the up-regulation of *Brca2* mRNA levels in ovariectomized animals treated with a combination of 17β-estradiol and progesterone was significantly lower than that observed for *Brca1*.

DISCUSSION

We have analyzed the spatial and temporal pattern of *Brca2* mRNA expression during fetal development, in adult tissues, in the mammary gland during postnatal development and in ovariectomized animals treated with various combinations of ovarian hormones. These studies suggest that *Brca2* is expressed during fetal development and in adult tissues within cellular compartments actively involved in proliferation and differentiation. This hypothesis is supported by the observation that *Brca2* mRNA expression in the breast is developmentally regulated and is found at highest levels in terminal end buds during puberty and differentiating alveoli during pregnancy. Moreover, this pattern of expression is consistent with the genetic identification of *BRCA2* as a tumor suppressor gene and suggests that this gene may play a role in the regulation of cellular proliferation and differentiation in the mammary gland and other tissues.

Coordinate and Differential Regulation of Brca1 and Brca2 Expression

A particularly intriguing finding of these studies is the striking degree to which *Brca2* and *Brca1* are temporally and spatially coexpressed at the mRNA level. *Brca1* and *Brca2* are expressed at similar relative levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, during fetal development and in multiple adult tissues, including breast, ovary, uterus, and small intestine, the spatial and temporal patterns of *Brca1* and *Brca2* expression are virtually indistinguishable. This simi-

mRNA expression shown in (A). *Brca1* mRNA expression is normalized to β-actin mRNA expression to correct for dilutional changes in signal intensity. The *Brca1*/actin ratio is defined as 1.0 for the 15-week virgin sample. (D) Phosphorimager quantitation of *Brca1* and *Brca2* mRNA expression levels in the mammary glands of age-matched adult male and female mice normalized to actin, and of the ratio of female:male mRNA expression for each of these genes. Mammary glands from approximately 130 females and 80 males were analyzed as multiple independently pooled samples. $^*P < 0.005$; $\ddagger P < 0.01$. Error bars represent the standard error of the mean. (E) *In situ* hybridization analysis of *Brca2* mRNA expression during postnatal mammary gland development. Bright-field and dark-field (antisense) photomicrographs of sections from mouse mammary glands at the indicated developmental time points hybridized to a *Brca2* antisense probe. No signal was detected over background in serial sections hybridized with a sense *Brca2* probe (data not shown). teb, terminal end bud; st, mammary stroma; d, epithelial duct; al, developing alveoli during pregnancy; lo, casein-secreting lobule during lactation or early regression.

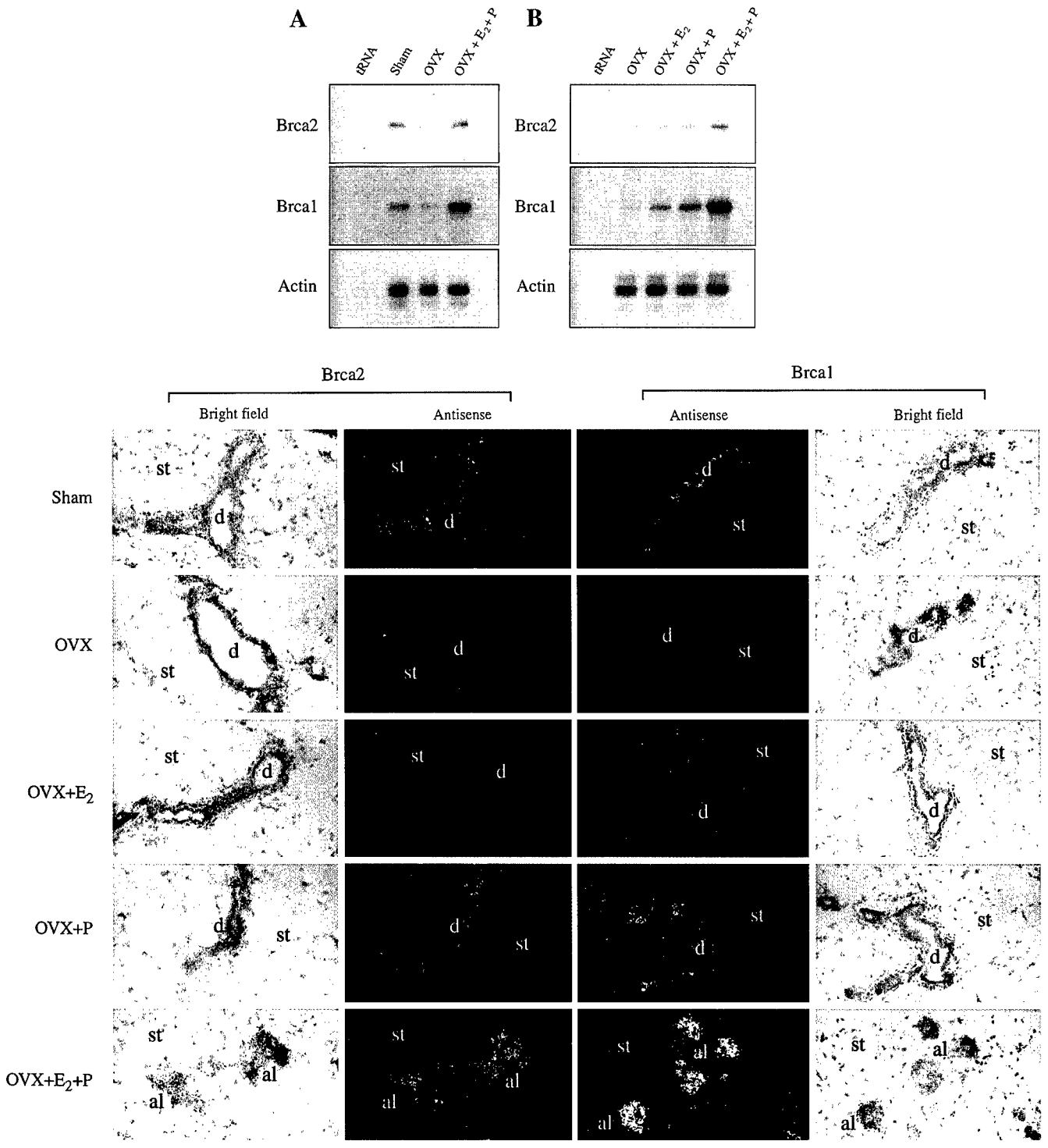

FIG. 7. Differential induction of *Brca1* and *Brca2* mRNA expression in the mammary gland by ovarian hormones. (A) *Brca2* expression is dependent on ovarian hormones. RNase protection analysis of *Brca2* and *Brca1* mRNA expression in the mammary glands of control and ovariectomized mice treated with estradiol and progesterone. Control animals (Sham) did not undergo surgery. Ovariectomized mice received either no hormone (OVX) or a combination of 17β-estradiol and progesterone (OVX + E2 + P) for 13 days prior to sacrifice. A ^{32}P-labeled antisense probe for β-actin was included in each reaction as an internal control. (B) *Brca2* mRNA is induced by estradiol and progesterone to a lesser extent than *Brca1*. RNase protection analysis of *Brca2* and *Brca1* mRNA expression in the mammary glands of ovariectomized mice treated with varying combinations of ovarian hormones. Ovariectomized mice received either no hormone (OVX),

larity is particularly evident during postnatal mammary gland development, as each of these putative tumor suppressor genes is up-regulated during puberty and pregnancy and as a result of parity. A potential basis for this similarity is provided by the observation that *Brca1* and *Brca2* expression are coordinately up-regulated in proliferating and differentiating mammary epithelial cells *in vitro* (Rajan *et al.*, 1996). These findings suggest that the mRNA expression of these two breast cancer susceptibility genes may be regulated by similar pathways and stimuli in multiple cell types. Given this striking similarity and the fact that mammary epithelial cells are similarly predisposed to transformation by inherited mutations in either *BRCA1* or *BRCA2*, it is tempting to speculate that *BRCA1* and *BRCA2* may have overlapping functions or may even act in concert. Nevertheless, the fact that germline mutations in *BRCA2* predispose carriers to increased risks of breast cancer despite the presence of presumably wild-type copies of *BRCA1*, and vice-versa, suggests that the functions of these two genes are not entirely redundant. This conclusion is supported by the fact that germline mutations in *BRCA1* and *BRCA2* result in distinct phenotypes with respect to the incidence of ovarian cancer, male breast cancer, and pancreatic cancer.

Given the remarkable similarities in the expression profiles of *Brca1* and *Brca2*, differences in the expression pattern of these two genes may be informative. Our studies demonstrate that *Brca1* and *Brca2* expression are differentially regulated in the breast by ovarian hormones. Specifically, *Brca1* expression is induced in the breast to a significantly greater extent than *Brca2* both during pregnancy and following estradiol and progesterone treatment of ovariectomized mice. Conversely, *Brca2* mRNA levels are markedly up-regulated in the testis during the first 4 weeks of life, whereas *Brca1* mRNA levels remain essentially unchanged. Taken together, these results indicate that *Brca1* and *Brca2* mRNA expression are differentially regulated by sex hormones. In this regard, it is interesting to note that mRNA expression in the mammary glands of female mice relative to male mice is significantly greater for *Brca1* than for *Brca2*. Presumably, this may reflect, at least in part, the more pronounced effect of ovarian hormones on *Brca1* expression. The finding that *Brca1* expression levels in the breast are markedly greater in females relative to males, whereas *Brca2* expression levels are more comparable, is particularly intriguing given that germline *BRCA2* mutations are associated with an increased risk of both male and female breast cancer, whereas the increased breast cancer risk associated with germline *BRCA1* mutations is restricted to females. These findings raise the possibility that the gender-specific differences in breast cancer risk associated with germline mutations in *BRCA1* versus *BRCA2* reflect the differential regulation of these genes by sex hormones.

Regulation of Brca1 and Brca2 Expression in Proliferating Cells

A central feature of the *in vivo* pattern of *Brca1* and *Brca2* expression is that each of these putative tumor suppressor genes is expressed at maximal levels in rapidly proliferating cells. This feature is consistent with *in vitro* observations that *Brca1* and *Brca2* mRNA expression are each up-regulated in rapidly proliferating cells, are down-regulated in response to serum deprivation, and are expressed in a cell cycle-dependent manner, peaking at the G1/S boundary (Chen *et al.*, 1996; Gudas *et al.*, 1996; Rajan *et al.*, 1996; Vaughn *et al.*, 1996a,b). Formally, these observations are consistent with a model in which *Brca1* and *Brca2* exert a positive effect on proliferation. Such a model would be compatible with studies demonstrating that mouse embryos bearing homozygous targeted mutations in *Brca1* exhibit a decrease in cellular proliferation (Hakem *et al.*, 1996; Liu *et al.*, 1996). Of particular interest is the observation that $p21^{WAF1/CIP1}$ expression is up-regulated in cells bearing homozygous deletions in *Brca1*, suggesting that *Brca1* may negatively regulate $p21^{WAF1/CIP1}$ expression (Hakem *et al.*, 1996). This hypothesis would be consistent with our observation that *Brca1* and $p21^{WAF1/CIP1}$ are expressed in several fetal tissues in a mutually exclusive pattern. Nevertheless, it is difficult to reconcile a model in which *Brca1* and *Brca2* positively regulate cellular proliferation with the genetic evidence that *BRCA1* and *BRCA2* are tumor suppressor genes and with the observations that overexpression of *BRCA1* inhibits proliferation while reduction in *BRCA1* expression promotes proliferation (Holt *et al.*, 1996; Rao *et al.*, 1996; Thompson *et al.*, 1995). Moreover, analysis of a third *Brca1* knockout model suggests that mouse embryos bearing homozygous targeted mutations in *Brca1* may exhibit an increase, rather than a decrease, in prolif-

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17β-estradiol alone (OVX + E2), progesterone alone (OVX + P), or a combination of 17β-estradiol and progesterone (OVX + E2 + P) for 20 days prior to sacrifice. A ^{32}P-labeled antisense probe for β-actin was included in each reaction as an internal control. (C) *In situ* hybridization analysis of *Brca2* and *Brca1* mRNA expression in mammary glands from ovariectomized mice treated with ovarian hormones. Bright-field and dark-field (antisense) photomicrographs of *in situ* hybridization analysis performed using ^{35}S-labeled antisense probes for *Brca2* or *Brca1* on frozen sections of mammary glands harvested from ovariectomized mice that treated with either no hormone (OVX), 17β-estradiol alone (OVX + E2), progesterone alone (OVX + P), or a combination of 17β-estradiol and progesterone (OVX + E2 + P). Control animals (Sham) did not undergo surgery. Sections shown for *Brca2* and *Brca1* antisense probes for each experimental treatment were taken from the same mammary gland of the same animal. To facilitate comparison, all dark-field photomicrographs shown were taken using identical shutter exposure times. No signal was detected over background in serial sections hybridized with sense *Brca2* or *Brca1* probes (data not shown). d, epithelial duct; st, stroma; al, developing alveolus.

eration in certain cell types (Gowen *et al.*, 1996). As such, whether *BRCA1* is a positive or negative regulator of proliferation in adult tissues remains unclear. One possibility is that the functions of *BRCA1* in embryonic tissues may differ from those in adult tissues. Regardless, the observation that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy, periods of development associated with both increases in cellular proliferation and increases in breast cancer risk in humans, raises the possibility that the induction of *Brca1* and *Brca2* expression is a protective response to proliferation (Rajan *et al.*, 1996; Boice and Monson, 1977; Lambe *et al.*, 1994; McGregor *et al.*, 1977; van Leeuwen *et al.*, 1994). Such a model would postulate that these putative tumor suppressor genes participate in a homeostatic regulatory loop in which proliferation results in the induction of *BRCA1* and *BRCA2* expression which, in turn, slow the rate of proliferation, or exert important checkpoint control functions, during these critical developmental stages. In fact, the observation that few somatic mutations in *BRCA1* or *BRCA2* have been identified in sporadic breast cancers suggests that the normal function of these genes may be restricted to specific developmental stages. To the extent that *BRCA1* and/or *BRCA2* may serve to control proliferation during developmental stages characterized by rapid growth, this model predicts that individuals possessing germline mutations in *BRCA1* and/or *BRCA2* may be particularly susceptible to early events in mammary carcinogenesis during pregnancy. Consistent with this model, recent epidemiological observations suggest that women with a positive family history of breast cancer may experience a significantly greater increase in breast cancer risk associated with their first pregnancy relative to women without a family history of breast cancer (Colditz *et al.*, 1996). Whether this observation is related to the loss or impairment of the normal function of *BRCA2* (and/or *BRCA1*) is unknown. Interestingly, *Brca2* mRNA levels appear to remain elevated in the mammary epithelium as a result of parity. We have previously made a similar observation for *Brca1* (Marquis *et al.*, 1995). These observations are consistent with the hypothesis that the mammary glands of both parous women and parous rodents are more differentiated relative to age-matched virgin controls (Russo *et al.*, 1982, 1992; Russo and Russo, 1993). As such, *Brca2* and *Brca1* expression levels may serve as molecular markers for the differentiated state of the mammary epithelium. Moreover, the prolonged elevation in expression of these breast cancer susceptibility genes in the mammary glands of parous mice may be related to the phenomenon of parity-induced protection against breast cancer in women who have undergone their first full-term pregnancy early in life (Marquis *et al.*, 1995; Lambe *et al.*, 1994; MacMahon *et al.*, 1970). Further studies of *BRCA1* and *BRCA2* function will undoubtedly serve as a foundation for understanding how their absence or mutation promotes carcinogenesis, as well as provide insight into their role in the developmental regulation of proliferation and differentiation in mammalian cells.

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Differential expression of the *neu* transgene in murine mammary tissues

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Abstract. The mammary glands of control FVB and mice with MTV-LTR promoted transgenes were stained using immunohistochemistry to detect *neu* expression. *Neu* expression in the terminal end buds of developing mammary glands and during early pregnancy in FVB mice was confirmed by *in situ* hybridization. *Neu* was expressed in all tumors from mice with the *neu* transgene but not in tumors expressing transforming growth factor α (TGFα) or polyoma virus middle T (PyV-MT). *Neu* was also expressed sporadically in non-neoplastic mammary cells of transgenic *neu* mice. However, most mammary cells expressing *neu* were dysplastic. The differential expression of the *neu* transgene has important implications for the interpretation of transgenic biology.

Introduction

Transgenic biology has provided important insight into the neoplastic process. The biological potential of a number of known and candidate oncogenes has been verified, and their expression in transgenic animals has led to the discovery of new oncogenes. Genetic knock outs have been utilized to prove the role of some tumor-suppressor genes. Recently, combinations of suppressor- and oncogenes have revealed complementary interactions (1).

Interpretations of tumorigenesis in transgenic mice have frequently assumed that the transgene is uniformly expressed in the target organ. However, the target organs of most transgenic animals exhibit normal growth and development until such time as other events or stimuli result in abnormalities; abnormalities which are often focal. This apparent inconsistency has been most extensively studied in the mouse mammary gland (2). It is now clear that oncogene expression is necessary but not sufficient in neoplastic development. Two lines of investigation serve as hypothetical models for this apparent paradox: molecular mutation and differential expression.

The molecular mutation explanation for these observations is that additional molecular alterations and interactions are required for malignant transformation. The work of Muller and his colleagues with mice bearing the *neu* transgene has provided evidence that, in at least some models, neoplastic progression involves further molecular alterations and the induction of additional genes (3). Muller has also demonstrated that PyV-MT mediated neoplastic transformation requires the presence of the *src* gene (4). These studies support a molecular mutation model of neoplastic progression.

The differential expression hypothesis is that neoplastic transformation may be the result of overexpression of a given transgene in certain subpopulations of cells. This hypothesis is supported by the observations of patchy, heterogeneous transgene expression of tyrosinase and lactoglobulin (5-7). Since the expression of these genes should be uniform throughout the organ, and since the patchy expression is not necessarily due to mosaicism, it is possible that similar heterogeneity of transgene expression might effect tumorigenesis in animals carrying oncogenes.

Support for differential transgene expression in mammary tumorigenesis was first provided by the observation that TGFα expression is limited to areas of cystic hyperplasia in MTV-TGFα transgenic mice (8). This idea was reinforced by an immunological study of *myc* expression in transgenic mice wherein immunohistochemical (IHC) expression was found only in dysplastic mammary glands and in tumors (9).

In the current study, we have examined the distribution of *neu*-related antigens and of mRNA using immunoperoxidase and *in situ* hybridization in order to localize *neu* expression

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in a variety of mouse mammary glands from various strains, and from different laboratories. These studies provide evidence that a combination of differential expression and molecular alteration is involved in neoplastic progression in the mouse mammary gland.

Materials and methods

Tissue specimens. Mammary tissue from 8 strains of MTV-LTR promoted transgenic mice from four labs (TGFα, TGFα*xneu* (10), activated *neu* (11), wild-type *neu* (c-*erb*B-2) (12), Polyoma Virus Middle T (PyV-MT) (13), and *ras* (13) and non-transgenic control FVB mice, with various histologic changes including tumors, hyperplasias/dysplasias, stages of lactation and regression, and mammary tissue from various developmental stages of FVB mice were received in paraffin blocks, in 10% neutral-buffered formalin, or fixed in Omnifix and post fixed in 70% ethanol. The TGFα*xneu* mice were bigenic animals that were the result of crosses between mice carrying the *neu* transgene and mice with the TGFα transgene (10). The formalin-fixed tissue was processed and embedded in paraffin using standard methods. Five to ten-micron thick tissue sections were cut and mounted on positively charged (treated with 3-aminopropyltriethoxysilane) slides. All blocks came from the archives of the U.C. Davis Transgenic Histopathology Laboratory.

Immunohistochemistry (IHC). Staining procedures were performed manually. Tissue sections were deparaffinized and hydrated in deionized water. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide in methanol and rinsed thoroughly with deionized water. Sections were rinsed with deionized water and phosphate-buffered saline (PBS), and incubated with normal horse serum to block nonspecific antibody binding. The primary polyclonal rabbit antisera against HER-2/*neu* (800-874-8667) from Ciba-Corning (Ciba Corning, Alameda, CA) was applied and incubated overnight at 4°C. The following day, the sections were washed in PBS and incubated with a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). After rinsing in PBS, sections were incubated with an avidin-biotin enzyme complex (Vector laboratories). Sections were rinsed in deionized water, counterstained in Harris hematoxylin, dehydrated through graded alcohol, cleared in xylene, and coverslipped. The photographs for Fig. 6 were digitized using Photoshop 3.05, balanced for contrast and brightness and printed on a Kodak 8650 dye sublimation printer.

In situ hybridization (ISH). In situ hybridization was performed as previously described (14) with the exception that mammary tissues were fixed with 4% paraformaldehyde in PBS and embedded in paraffin prior to sectioning. Sections were cut at 5 μm, heat fixed, deparaffinized in xylene, then sequentially hydrated and dehydrated in graded concentrations of ethanol. Probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP and the T7 and SP6 RNA polymerase promoters of a template derived from a mouse *neu* cDNA corresponding to amino acids 847-914 of rat *neu*. This probe is specific for *neu* sequences. Sense cDNA was used as an *in situ* hybridization negative control. To facilitate comparison, all dark field photomicrographs shown were taken using identical shutter exposure times. The photographs were digitized using Photoshop 3.05, balanced for contrast and brightness and printed on a Kodak 8650 dye sublimation printer.

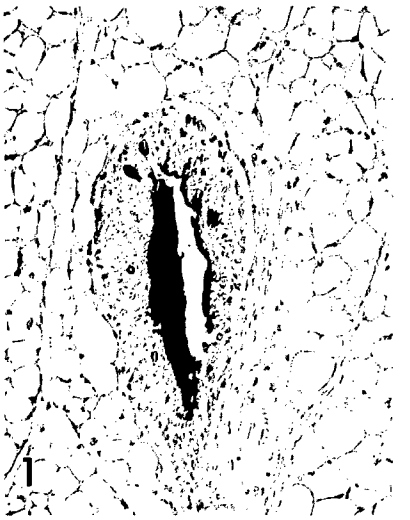

Figure 1. Photomicrograph illustrating the distribution of anti-*neu* staining in the terminal end bud of a 7-week old female FVB mouse. Note that the stain is along the luminal margin (x360).

Northern hybridization analysis. Poly(A)$^+$ RNA for each developmental time point was isolated from snap-frozen mammary glands harvested from approximately 20 age-matched mice (15). Northern hybridization analysis was performed as previously described (14). The cDNA fragment of mouse *neu* used as a probe for Northern hybridization contained sequences corresponding to amino acids 847-914 of rat *neu*.

Results

Normal mammary gland. Non-transgenic wild-type: Normal FVB females terminated at various stages of their growth and development, including puberty, pregnancy and lactation were studied. *Neu*-related antigens were detected in scattered cells of the terminal end buds of the mitotically active, pubertal mammary gland (Fig. 1) (Table I). The staining was primarily membrane, and concentrated on the differentiating cells of the luminal borders. Staining was not seen on the proliferative stem cells, or the 'cap cells'. The positive staining cells did not have morphologic features that distinguished them from the non-staining cells. The duct lumens also contained intensely staining *neu*-positive material. The fully mature, mitotically quiescent mammary gland did not express the antigen. *Neu*-related antigens were detected at the luminal margins of proliferating end buds at early and mid pregnancy when the mammary tissue is again undergoing proliferative activity, but not in the lactating or regressing mammary gland.

To determine whether the observed staining pattern reflected *neu* mRNA levels, *in situ* hybridization (ISH) was

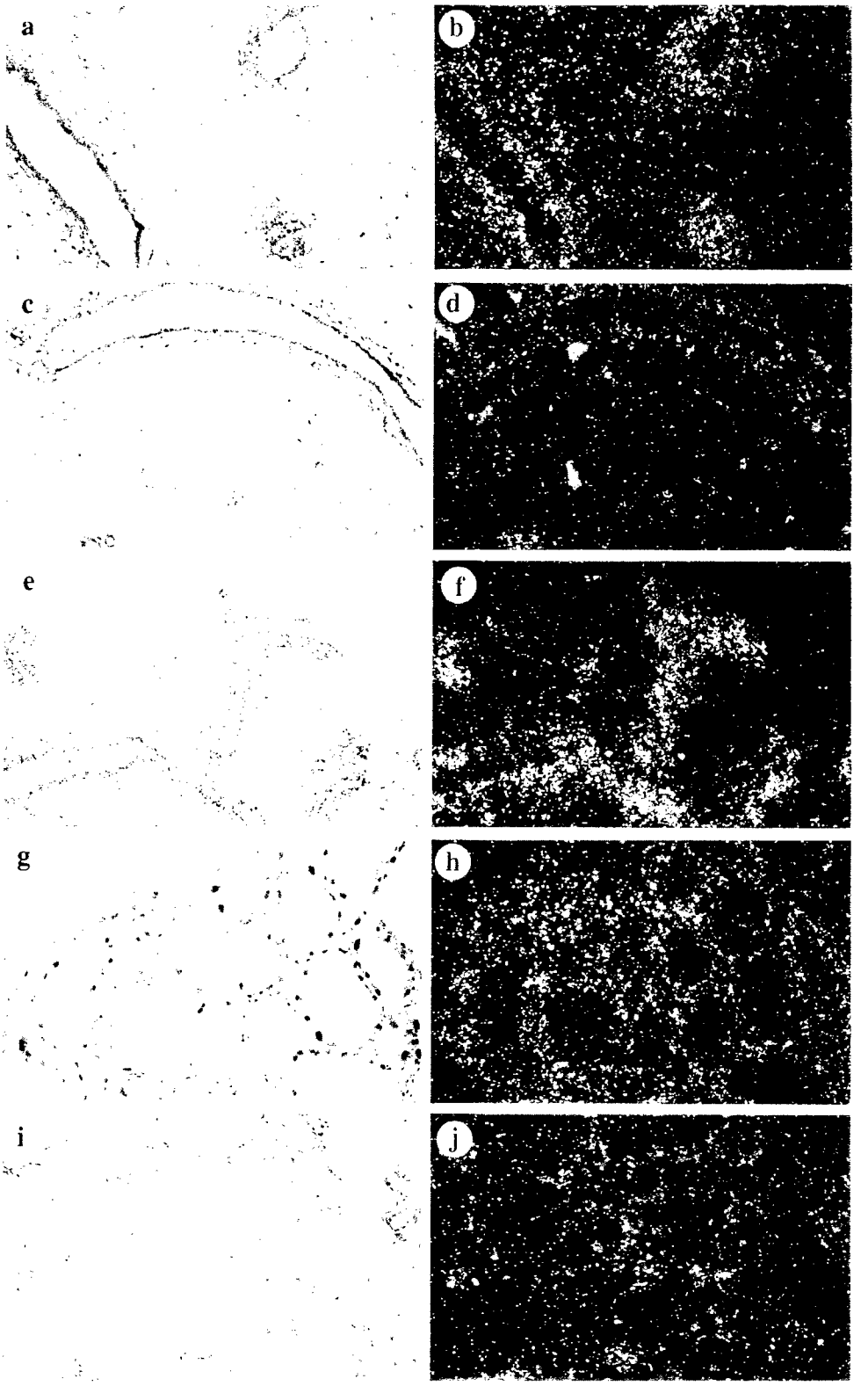

Figure 2. *In situ* hybridization analysis of *neu* mRNA expression during postnatal mammary gland development in the FVB mouse. Bright field (a, c, e, g, i) and dark field (b, d, f, h, j) photomicrographs of sections from female mouse mammary glands at the indicated developmental stages hybridized with an antisense *neu* probe: (a, b) 6-week old virgin; (c, d) 16-week old virgin; (e, f) day 7 pregnancy; (g, h) lactation; (i, j) 16-week old parous status post 4 weeks post-lactational regression. Note high levels of *neu* mRNA expression in terminal end buds during puberty and in ducts and developing alveoli during pregnancy (x300).

performed on tissue blocks representing these same developmental time points. In addition, Northern hybridization analysis of *neu* mRNA expression was examined during postnatal development in the FVB mouse. Using an antisense probe, high levels of *neu* mRNA expression were detected in terminal end buds during puberty and in ducts and developing alveoli during pregnancy (Fig. 2). No hybridization was detected using a control sense probe. Northern blots

Table I. *Neu*-positive immunohistochemical staining.

Genotype	Mammary gland morphology						
	Nullparous	Pregnancy	Lactating	Regressing	Tumor	Hyperplasia/ Dysplasia	Total mice
FVB	0/14*	2/3	0/1	0/2			20
neu					2/2	1/1	3
c-*neu* (c-*erb*B-2)			2/2	0/1	13/13	2/2	15
neu+/TGFα-			1/1	1/1	4/4	1/1	5
neu+/TGFα+		5/5	4/4	1/6	7/7		17
neu-/TGFα+			0/2				2
TGFα					0/6		6
PyV-MT					0/4	0/4	4
ras					0/2		2

*The number of *neu* positive mammary tissues per total animals.

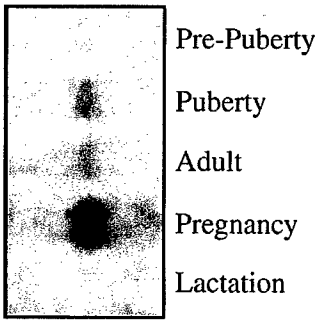

Figure 3. Northern hybridization analysis of *neu* mRNA expression during postnatal development in the FVB mouse. Poly(A)$^+$ RNA was isolated from the mammary glands of FVB mice harvested at the indicated developmental stages. Note the modest up-regulation of *neu* mRNA levels during puberty and marked up-regulation of *neu* mRNA levels during early pregnancy.

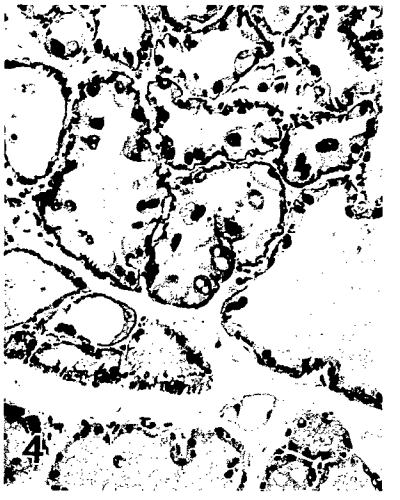

Figure 4. Photomicrograph illustrating the distribution of anti-*neu* staining in the alveoli of a lactating *neu* mouse. Note that the stain is along the basolateral margins and that adjacent mammary gland does not stain. The non-staining alveolar cells have smaller nuclei and cells (x360).

showed modest up-regulation of *neu* mRNA levels during puberty and marked up-regulation of *neu* mRNA levels during early pregnancy but down-regulation during lactation (Fig. 3). The up-regulation correlated to the increased IHC staining and ISH silver grains in the corresponding time periods.

Transgenic: Lactating mammary glands of selected *neu* transgenics were studied. The positive lactating cells showed an intense stain at the basal and lateral membranes. The apical membranes did not stain (Fig. 4).

The immunohistochemical stain was not distributed uniformly throughout the mammary gland. Considerable inter- and intra-alveolar variation was present. In some cases, the positive cells were found scattered throughout the gland. The scattered pattern of expression could be found involving entire acini or individual cells. *Neu*-negative cells could be found immediately adjacent to the positive staining cells (Fig. 4). In many sections, the *neu*-positive cells were found primarily in small, hyperplastic clusters quite separate from the more normal-appearing architecture of the lactating glands. In other cases, large patches of acini were IHC positive for *neu* antigens along their basal and lateral membranes.

Interestingly, the *neu*-positive cells tended to have much larger, more pleomorphic nuclear morphology than the *neu*-negative cells (Fig. 4). However, not all cells with pleomorphic nuclei were *neu*-positive, and not all morphologically typical cells were *neu*-negative.

Staining of regressing transgenic mammary tissue was variable (Table I). The overall quantity of IHC positive cells was low. However, *neu* staining cells found in regressing

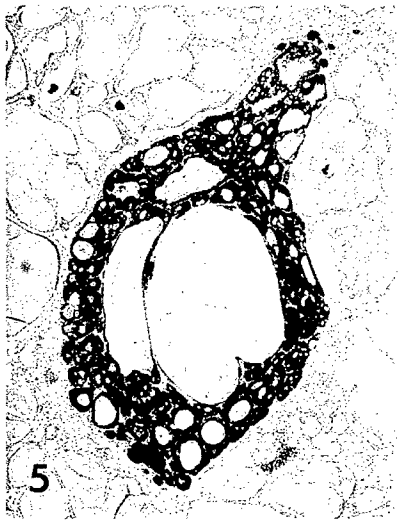

Figure 5. Photomicrograph illustrating the distribution of anti-*neu* staining in a lactating mammary gland of a multiparous TGFα*xneu* female. Note the dense island of stain surrounded by non-staining mammary tissues (x90).

Figure 7. Photomicrograph illustrating the distribution of anti-*neu* staining in the tumor of a animal with the *neu* transgene. Note that the stain is along the cell membrane creating a honeycomb pattern (x360).

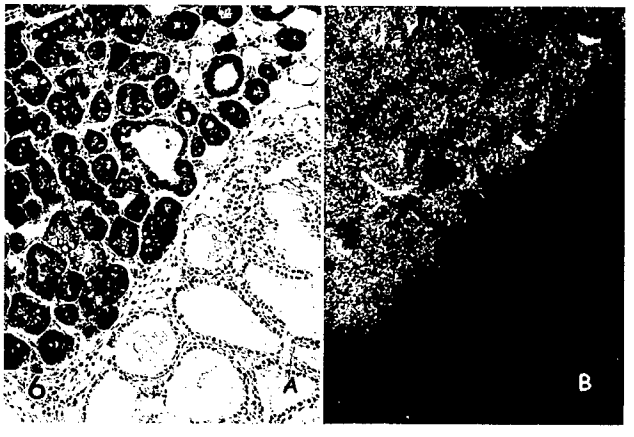

Figure 6. A, Photomicrograph illustrating the distribution of anti-*neu* staining in an area of atypical hyperplasia in a female TGFα*xneu* mouse. While the whole mammary gland is hyperplastic the *neu* positive tissue has smaller lumina and larger cells (x125). B, A photomicrograph showing a darkfield image of *in situ* hybridization of a similar area in the same tissue block. Note that the greatest grain intensity is over the *neu* positive cells (x125).

mammary tissue were in clusters of tall columnar cells. Although not dysplastic, these cells stood out against the IHC negative regressing gland.

Mammary hyperplasias. Transgenic non-neu: None of the PyV-MT, TGFα or the *ras* transgenic hyperplastic mammary glands stained with the anti-*neu* antibody.

Transgenic neu: The *neu* transgenic, hyperplastic mammary glands revealed small foci of nodular hyperplasias with occasional areas of cytologic atypia. The atypical foci were most dramatic in the bigenic TGFα*xneu* (Figs. 5 and 6A). These animals, expressing both the TGFα and the *neu* transgenes exhibited accelerated tumorigenesis (10). As predicted for mice with overexpression of both the ligand and the receptor, the mammary glands were hyperplastic and had foci of abnormal cells (10). Most of the cells within the abnormal areas stained for *neu* (Figs. 5 and 6A). Although the staining was patchy, most of the atypical cells stained. *Neu*-positive cells were adjacent to non-staining cells, and groups of positive staining cells occurred adjacent to groups of non-staining cells. The staining was predominately membrane, and was concentrated at the base and the lateral membrane edges. The adjacent hyperplastic but non-dysplastic mammary tissue did not stain. The patchy pattern of IHC staining corresponded to the density of silver grains found by ISH (Fig. 6B).

Mammary tumors. Transgenic non-neu: None of the non-*neu* TGFα, *ras* or Polyoma Virus Middle T transgenic tumors stained for the *neu* antigen (Table I). Further, the ISH did not detect *neu* mRNA in these tumors.

Transgenic neu: Neu-related tumors are generally characterized by solid, nodular masses of tumors cells with peripheral palisading (16). The nuclei are of intermediate size, round, and have a dispersed chromatin pattern and small nucleoli (16). All of the tumors from *neu*-transgenic mice stained for *neu*. The staining was predominately membrane, not always uniformly distributed throughout the tumor (Fig. 7). The intensity of the stain was highest at the periphery of the nests. The ISH resulted in the highest density of silver grains over all of the *neu*-related tumors.

Bigenic neu: Transgenic mice TGFα*xneu* develop tumors with the architectural and morphologic characteristics of *neu* (10). These tumors demonstrated a similar staining pattern as the *neu* tumors. *Neu*-positive lines from two additional sources also confirmed the above findings (unpublished observations).

Discussion

The studies reported above document, using immunohistochemistry and *in situ* hybridization, that the *neu* transgene is

differentially expressed in the mammary glands, mammary hyperplasias and mammary tumors of mice carrying the *neu* transgene. These observations should affect our understanding and interpretation of transgenic biology.

The early interpretations of tumorigenesis in transgenic mice tacitly assumed that the oncogene or transgene was uniformly expressed in the affected organ. Indeed, biochemical evidence has clearly demonstrated that the target organs have more expression of the transgene than normal or non-target organs (2,16). This has led to the assumption that the transgene is uniformly 'overexpressed' in the target organ. Massive hyperplasias found in some transgenic animals have supported the idea of 'simultaneous transformation' (17). Such interpretations have had a profound effect on modeling of tumorigenesis in transgenic animals.

The *neu* transgenic mouse is one such system that seemed to lend itself to a single step transformation hypothesis (2). The *int*-2 (fgf3) and TGFα transgenes also involve massive, uniform hyperplasias (8,18). However, the hyperplasia cannot be serially transplanted into ectopic sites (19,20). This suggests that *int*-2 and TGFα mediated 'simultaneous transformation' is non-malignant and that malignant transformation is a multi-step phenomenon.

In the studies reported here, dysplastic and neoplastic mammary tissue of mice expressing the *neu* transgene were examined. The bigenic mice expression both in the TGFα and the *neu* transgenes were of particular interest because the combination simultaneously express the growth factor-ligand and a receptor. The combination accelerates tumorigenesis (10). The tumors developing in these bigenic animals most frequently resembled the *neu* type of tumor (10). The most obvious morphological progression was observed in these animals (10).

Our studies clearly demonstrated that the *neu* transgene is not uniformly expressed in the target tissue. Rather, the transgene is differentially expressed in certain sub-populations of mammary cells. These cells, even in the lactating mammary gland, appear to have larger, more pleomorphic nuclei than the cells which do not express the transgene. Since all of the tumors resulting from the introduction of the *neu* transgene stain for the *neu* antigen and higher levels of *neu* mRNA, it is reasonable to assume that the tumors arise from the population subset that is expressing the mRNA and the antigen. Possible explanations for the heterogeneous distribution of *neu* antigen include mosaicism, sex linkage, transgene instability, expression instability and the transgene integration locus (7).

Differential expression has also been detected in the mammary glands of animals bearing the TGFα and the *myc* transgenes (8,9). These observations, coupled with the description of heterogeneity of transgene expression in lactoglobulin and tyrosinase mice, suggest that the phenomenon of differential transgene expression may occur in most transgenics (5-7). Further, the phenomena observed in our study appeared in many different strains from different founders and in different generations. Based on these and other experimental observations, mosaicism, and sex linkage of the transgene seem to be unlikely explanations and may be eliminated as possible explanations for the heterogeneous expression of *neu*. Further studies are needed to explore the alternate possibilities of transgene instability, expression instability and the effect of the transgene integration locus (7).

This study also shows that a *neu*-related antigen is expressed during normal growth and development and during pregnancy in FVB mice. In contrast to the evidence presented in the developing rat mammary gland, our Northern blot and *in situ* hybridization results indicate that both the mRNA and the antigen are down-regulated during lactation in the normal mouse mammary gland (21). Our observations raise several important questions, including the role this proto-oncogene plays during normal development and pregnancy, and how it is involved in malignant transformation.

These observations are important for the interpretation of tumorigenesis in any transgenic animal. Models must now account for the fact that transgenes are not uniformly expressed in the target tissue, and that their proto-oncogenes may also be expressed during development in normal mice. This raises the possibility that overexpression of an oncogene at a cellular level can, in and of itself, be sufficient for the initial step in tumorigenesis, perhaps as cellular promoters. The rate of tumorigenesis could be related to the proportion of cells in the target organ which overexpress the transgene. Such observations confound the interpretation of the outcome when two or more transgenes are combined through crossing or backcrossing monogenic strains. Differential expression might also influence the phenotype in transgenic models testing cytokines or other immunologically related genes.

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Protein Kinase Expression during Murine Mammary Development

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ABSTRACT

The susceptibility of the mammary gland to carcinogenesis is influenced by its normal development, particularly during developmental stages such as puberty and pregnancy that are characterized by marked changes in proliferation and differentiation. Protein kinases are important regulators of proliferation and differentiation, as well as of neoplastic transformation, in a wide array of tissues, including the breast. Using a RT-PCR-based cloning strategy, we have identified 41 protein kinases that are expressed in breast cancer cell lines and in the murine mammary gland during development. The expression of each of these kinases was analyzed throughout postnatal mammary gland development as well as in a panel of mammary epithelial cell lines derived from distinct transgenic models of breast cancer. Although the vast majority of protein kinases isolated in this screen have no currently recognized role in mammary development, the majority of kinases examined exhibit developmental regulation. After clustering kinases on the basis of similarities in their temporal expression profiles during mammary development, multiple distinct patterns of expression were observed. Analysis of these patterns revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. Interestingly, several protein kinases whose expression has previously been reported to be restricted to tissues other than the mammary gland were isolated in this screen and found to be expressed in the mammary gland. In aggregate, these findings suggest that the array of kinases participating in the regulation of normal mammary development is considerably broader than currently appreciated.

INTRODUCTION

Numerous epidemiologic and animal studies analyzing the impact of reproductive events such as puberty, pregnancy and parity on early events in carcinogenesis suggest that the developmental state of the breast plays a critical role in the determination of breast cancer risk (Lambe *et al.*, 1994; MacMahon *et al.*, 1970; MacMahon *et al.*, 1982; Newcomb *et al.*, 1994; Russo and Russo, 1978; Russo and Russo, 1987). This implies an intrinsic relationship between the process of carcinogenesis and normal pathways of differentiation and development in the breast. Therefore, understanding the mechanisms by which reproductive events influence breast cancer susceptibility will undoubtedly require an improved understanding of normal mammary development, particularly with respect to genes that control mammary proliferation and differentiation.

Protein kinases represent the largest class of genes known to regulate differentiation, development and carcinogenesis in eukaryotes. Therefore, we have chosen to study members of this family of regulatory proteins as one approach to elucidating the relationship between development and carcinogenesis in the breast. Several protein kinases have been implicated in the development of breast cancer either in humans or in rodent model systems. For instance, the EGF receptor and *ErbB2/HER2* are each amplified and overexpressed in subsets of highly aggressive breast cancers, and these molecules may thereby provide prognostic information relevant to clinical treatment and outcome (Klijn *et al.*, 1993; Slamon *et al.*, 1987; Slamon *et al.*, 1989). Furthermore, overexpression of specific protein kinases, or of ligands for protein kinases, in the mammary epithelium of transgenic animals results in neoplastic transformation (Cardiff and Muller, 1993; Guy *et al.*, 1994; Muller *et al.*, 1990; Muller *et al.*, 1988). Finally, by analogy with hematopoiesis, some protein kinases are likely to be expressed in a lineage-restricted manner in the breast, and as such may provide insight into biologically meaningful subpopulations of cells (Dymecki *et al.*, 1990; Siliciano *et al.*, 1992; Tsukada *et al.*, 1993). These findings suggest that further analysis of protein kinase function may reveal significant features of the relationship between development and

carcinogenesis in the breast, as well as provide insight into how the decision to proliferate or differentiate is made in mammary epithelial cells.

In light of the importance of this class of regulatory molecules, we initiated a systematic study of the role of protein kinases in mammary gland development and carcinogenesis. Examination of 1,450 cDNA clones generated using a RT-PCR-based screening strategy identified 41 protein kinases, including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel. The expression of these kinases was subsequently examined during defined stages in mammary development and in a panel of mammary tumor cell lines derived from distinct transgenic models of breast cancer. Our findings reveal an ordered series of protein kinase expression patterns that occur during each of the stages of postnatal mammary development, suggesting that these molecules may be important regulators of this process.

MATERIALS AND METHODS

Cell Culture

Mammary epithelial cell lines were derived from mammary tumors or hyperplastic lesions that arose in MMTV-*c-myc*, MMTV-*int-2*, MMTV-*neu*/NT or MMTV-Ha-*ras* transgenic mice and included: the *neu* transgene-initiated mammary tumor-derived cell lines, SMF, NAF, NF639, NF11005, and NK-2; the c-*myc* transgene-initiated mammary tumor-derived cell lines, 16MB9a, 8Ma1a, MBp6, M158, and M1011; the Ha-*ras* transgene-initiated mammary tumor-derived cell lines, AC816, AC236, and AC711; the *int*-2 transgene-initiated hyperplastic cell line, HBI2, and the *int*-2 transgene-initiated mammary tumor-derived cell line, 1128 (Morrison and Leder, 1994). Additional cell lines were obtained from ATCC and included NIH3T3 cells and the nontranformed murine mammary epithelial cell lines, NMuMG and CL-S1. All cells were cultured under identical conditions in DMEM medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Animals and Tissues

FVB mice were housed under barrier conditions with a twelve hour light/dark cycle. The mammary glands from between ten and forty age-matched mice were pooled for each developmental point. Mice for pregnancy points were mated at 4-5 weeks of age. Mammary gland harvest consisted in all cases of the number three, four, and five mammary glands. The lymph node embedded in the number four mammary gland was removed prior to harvest. Tissues used for RNA preparation were snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT medium and frozen in a dry ice/isopentane bath.

Construction and Analysis of Kinase-specific cDNA Libraries

Kinase-specific cDNA libraries were constructed using mRNA prepared from the mammary glands of mice at specified stages of development and from a panel of mammary epithelial cell

lines. Specifically, total RNA was prepared from the mammary glands of either 5 week old nulliparous female mice or parous mice that had undergone a single pregnancy followed by 21 days of lactation and 2 days of postlactational regression. Total RNA was also prepared from the mammary epithelial cell lines NMuMG, CL-S1, HBI2, SMF, 16MB9a, AC816 and 1128, described above.

First-strand cDNA was generated from each of these nine sources of RNA using the cDNA Cycle kit according to manufacturer's directions (Invitrogen). These were amplified using degenerate oligonucleotide primers corresponding to conserved regions in kinase catalytic subdomains VI and IX. The degenerate primers, PTKIa (5'-GGGCCCGGATCCAC(A/C)G (A/G/C/T)GA(C/T)(C/T)-3') and PTKIIa (5'-CCCGGGGAATTCCA(A/T)AGGACCA(G/C)AC (G/A)TC-3'), have previously been shown to amplify a conserved 200 bp portion of the catalytic domain of a wide variety of tyrosine kinases (Wilks, 1989; Wilks, 1991; Wilks *et al.*, 1989). Two additional degenerate oligonucleotide primers, BSTKIa (5'-GGGCCCGGATCC(G/A)T(A/G)CAC (A/C)G(A/G/C)GAC(C/T)T-3') and BSTKIIa (5'-CCCGGGGAATTCC(A/G)(A/T)A(A/G)CTCC A(GC)ACATC-3') that differed from PTKIa and PTKIIa were also designed for this study. Restriction sites, underlined in the primer sequences, were generated at the 5' (*Apa*I and *Bam*HI) and 3' (*Xma*I and *Eco*RI) ends of the primer sequences.

Each cDNA source was amplified in three separate PCR reactions using pairwise combinations of either the PTKIa/PTKIIa, BSTKIa/BSTKIIa, or BSTKIa/PTKIIa degenerate primers. Following 5-min denaturation at 95°C, samples were annealed at 37°C for 1 min, polymerized at 63°C for 2 min, and denatured at 95°C for 30 sec for 40 cycles. The resulting ~200 bp PCR products were purified from low-melt agarose (BMB), ligated into a T-vector (Invitrogen), and transformed in *E. coli*. Following blue/white color selection, approximately 50 transformants were picked from each of the 27 PCR reactions (three PCR reactions for each of nine cDNA sources) and were subsequently transferred to gridded plates and replica plated. In total, 1,450 transformants were analyzed.

Dideoxy sequencing of 100 independent transformants was performed, resulting in the identification of 14 previously described tyrosine kinases. In order to identify and eliminate additional isolates of these kinases from further consideration, filter lifts representing the 1350 remaining transformants were hybridized individually to radiolabeled DNA probes prepared from each of the fourteen initially isolated kinases. Hybridization and washing were performed as described at final washing conditions of 0.1x SSC/0.1% SDS at 70°C that were demonstrated to prevent cross-hybridization between kinase cDNA inserts (Marquis *et al.*, 1995). In this manner, 887 tranformants were identified containing inserts from the 14 tyrosine kinases that had initially been isolated. Identifications made by colony hybridization were consistent with those made directly by DNA sequencing.

The remaining 463 transformants were screened by PCR using T7 and SP6 primers to identify those containing cDNA inserts of a length expected for protein kinases. 172 tranformants were found to have cDNA inserts between 150 and 300 bp in length and were subject to further analysis by successive rounds of dideoxy sequencing and colony lift hybridization. This resulted in the identification of 27 additional protein kinases.

Individual clones were sequenced using the Sequenase version 2 dideoxy chain termination kit (U.S. Biochemical Corp.). Putative protein kinases were identified by the DFG consensus located in catalytic subdomain VI. DNA sequence analysis was performed using MacVector 3.5 and the NCBI BLAST server.

RNA Preparation and Analysis

RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 µl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1.0% LE agarose gel and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using ^{32}P-labeled cDNA probes corresponding to

catalytic subdomains VI-IX of each protein kinase that were generated by PCR amplification of cloned catalytic domain fragments (Marquis *et al.*, 1995). In all cases calculated transcript sizes were consistent with values reported in the literature.

In Situ Hybridization

In situ hybridization was performed as described (Marquis *et al.*, 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the sequences used for Northern hybridization analysis.

RESULTS

Identification of Protein Kinases Expressed in the Murine Mammary Gland

As an initial step in studying the role of protein kinases in regulating mammary proliferation and differentiation, we designed a screen to identify protein kinases expressed in the mammary gland and in breast cancer cell lines. An RT-PCR cloning strategy was employed that relies on the use of degenerate oligonucleotide primers corresponding to conserved amino acid motifs present within the catalytic domain of protein tyrosine kinases (Wilks, 1989; Wilks *et al.*, 1989). RNA prepared from nine different sources was used as starting material for the generation of kinase-specific cDNA libraries. These sources included mammary glands from 5 week-old nulliparous mice undergoing puberty and parous mice at day 2 of involution, as well as a panel of seven murine mammary epithelial cell lines. Cell lines consisted of two nontransformed mammary epithelial cell lines in addition to five cell lines derived from tumors and hyperplasias that arose in the mammary glands of MMTV-*neu*, MMTV-*c-myc*, MMTV-Ha-*ras*, or MMTV-*int2* transgenic mice (Leder *et al.*, 1986; Muller *et al.*, 1990; Muller *et al.*, 1988; Sinn *et al.*, 1987).

First-strand cDNA prepared from each of these sources was independently amplified using the previously described degenerate oligonucleotide primers, PTKI and PTKII, that encode conserved amino acid motifs within catalytic subdomains VIb and IX (Hanks and Quinn, 1991; Wilks, 1991; Wilks *et al.*, 1989). In an effort to isolate a broad array of protein kinases, two additional degenerate oligonucleotide primers, BSTKI and BSTKII, were designed for use in this screen that are also directed against subdomains VIb and IX, but which differ in nucleotide sequence. Degenerate oligonucleotide primers were used in three pairwise combinations (PTKI/PTKII, BSTKI/BSTKII, and BSTKI/PTKII) to amplify first-strand cDNA from each of the 9 sources. The resulting 150-300 bp PCR products from each amplification were subcloned into a plasmid vector. Approximately 50 bacterial transformants from each of the 27 PCR reactions were replica plated and screened by a combination of DNA sequencing and colony lift hybridization in order to identify the protein kinase from which each subcloned catalytic domain fragment was derived.

A total of 1,450 bacterial transformants were analyzed by this approach. Of these, greater than 70% contained protein kinase cDNA inserts as determined by hybridization and sequencing. Analysis of these clones resulted in the identification of 33 tyrosine kinases and 8 serine/threonine kinases (Table I). The 19 receptor tyrosine kinases and 14 cytoplasmic tyrosine kinase isolated accounted for all but 33 of the 1,056 kinase-containing clones. The remaining clones were derived from 8 serine/threonine kinases, seven of which were represented by a single clone each, including each of the novel kinases isolated in this screen. Approximately half of the 41 kinases were isolated more than once, and most of these were isolated from more than one tissue or cell line (Table I and data not shown). Eight tyrosine kinases, including *Jak2*, *Fgfr1*, *EphA2*, *Met*, *Igf1r*, *Hck*, *Jak1* and *Neu* accounted for 830 (79%) of all clones analyzed (Table I). Conversely, eighteen kinases (44%) were represented by a single clone each, suggesting that further screening of cDNA libraries derived from these tissues and cell lines may yield additional kinases. The number of clones isolated for each kinase presumably reflects a combination of mRNA abundance and extent of homology to the oligonucleotide primers used in the amplification reaction.

Three novel protein kinases were identified in this screen, designated *Bstk1*, *2* and *3*. Each of these kinases contains the amino acid motifs characteristic of serine/threonine kinases (Fig. 1). *Bstk1* was isolated from a mammary epithelial cell line derived from a tumor that arose in an MMTV-*neu* transgenic mouse, and is most closely related to the *SNF1* family of serine/threonine kinases. Additional cDNAs encoding *Bstk1* have subsequently been isolated (Korobko *et al.*, 1997 and Gardner, unpublished results). *Bstk2* and *Bstk3* were each isolated from the mammary glands of mice undergoing early postlactational regression. *Bstk2* exhibits highest homology to kinases recently identified in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* and appears to represent the first vertebrate member of a new family of mammalian protein kinases (Kurioka *et al.*, 1998; Ligos *et al.*, 1998; Stairs *et al.*, 1998). *Bstk3* is most closely related to calcium/calmodulin-dependent protein kinase I, and full-length isoforms have subsequently been identified in the mouse and rat (Yokokura *et al.*, 1997 and Gardner, unpublished results).

Expression of Protein Kinases in Mammary Epithelial Cell Lines

As a first step in investigating the range of expression patterns for the 41 protein kinases isolated in this screen, we determined kinase expression profiles in a panel of 18 murine mammary epithelial cell lines (Fig. 2) These included 14 cell lines derived from independent tumors arising in transgenic mice expressing either the *neu, c-myc, H-ras*, or *int-2* oncogenes under the control of the MMTV LTR; one cell line derived from the hyperplastic mammary epithelium of an MMTV-*int*-2 transgenic mouse, and three nontranformed, non-transgenic mammary epithelial cell lines (Fig. 2) (Leder *et al.*, 1986; Morrison and Leder, 1994; Muller *et al.*, 1990; Muller *et al.*, 1988; Sinn *et al.*, 1987). Kinase expression was also investigated in NIH 3T3 fibroblasts in order to identify those kinases that might be expressed in a mesenchymal or epithelial-specific manner. All cell lines were grown under identical conditions and were harvested while actively proliferating.

Of the 41 kinases isolated in this screen, 25 were found to be ubiquitously expressed in the epithelial cell lines examined (Fig. 2 and data not shown). Steady-state mRNA levels for 11 of these ubiquitously expressed kinases exhibit relatively little variation among cell lines. These include *Tyk2, Neu, Ryk, Plk, Csk, Akt1, A-Raf, Prkmk3,* and the insulin receptor. Steady-state mRNA levels for the remaining 14 ubiquitously expressed kinases vary considerably among cell lines. These include the receptor tyrosine kinases, *Egfr, Igf1R, Met, Tyro3, EphA2, EphA7, and EphB3*, as well as the cytoplasmic tyrosine kinases *Jak1, Jak2, c-Abl, c-Src, Lyn and Tec,* and the serine/threonine kinase, *SLK.*

In contrast, mRNA expression of 11 of the kinases examined was detectable in only a subset of epithelial cell lines. These kinases range from those that are expressed within the majority of cell lines tested, such as *Fgfr1, Fyn, Axl,* and *Mlk1*, to kinases that are expressed in only a minority of these cell lines, such as *Tyro10, c-Fes, c-Kit,* and *Flt3*, (Fig. 2 and data not shown). Within this latter group of kinases, *Ron, Srm,* and *Hck* are expressed at detectable levels in only a single mammary epithelial cell line each (Fig. 2 and data not shown). Interestingly, both *EphB3* and *c-Kit* are preferentially expressed in tumor cell lines derived from MMTV-*neu* and MMTV-Ha-*ras* transgenic animals as compared to cell lines derived from MMTV-c-*myc* and MMTV-*int-2*

transgenic animals. Similar patterns of expression in this panel of cell lines have previously been reported for Protein Tyrosine Phosphatase ε and other molecules (Elson and Leder, 1995; Morrison and Leder, 1994).

Additional patterns of expression were observed that presumably reflect cell-type specificity. For instance, *Mlk1* is expressed in all cell lines except for NIH 3T3 fibroblasts and for MBp6, the sole mammary cell line that does not express the epithelial marker, cytokeratin 18. The resulting supposition that *Mlk1* expression is epithelial-specific was subsequently confirmed by *in situ* hybridization (Fig. 8). By comparison with *Mlk1*, *Tyro10* exhibited an inverse pattern of expression with steady-state levels of mRNA detectable only in NIH 3T3, MBp6, and the int-2-initiated tumor cell line, 1128, suggesting that this kinase is preferentially expressed in stromal as compared to epithelial cells. This hypothesis was also confirmed by *in situ* hybridization (Fig. 10E-H). Similarly, expression in mammary epithelial cell lines was detected neither for *Tie1* nor *Tie2*, each of which has been shown to be expressed in an endothelial-specific manner, nor for *MuSK*, whose expression is restricted to muscle (Ganju *et al.*, 1995; Partanen *et al.*, 1992; Sato *et al.*, 1993; Valenzuela *et al.*, 1995b).

Surprisingly, expression of the receptor tyrosine kinases, *EphB3/Hek2* and *EphA7/Hek11/Ehk3*, was demonstrated in all of the mammary epithelial cell lines examined, despite the fact that expression of these kinase has been reported to be restricted primarily to the central nervous system (Aasheim *et al.*, 1997; Adams *et al.*, 1999; Bergemann *et al.*, 1998; Fox *et al.*, 1995; Krull *et al.*, 1997; Valenzuela *et al.*, 1995a). Similarly, despite previous reports that expression of each of the nonreceptor tyrosine kinases *Lyn*, *Tec* and *Hck*, is restricted primarily to cells of hematopoietic origin, *Lyn* and *Tec* expression were detected in all 18 mammary epithelial cell lines tested, and *Hck* expression was detected in two mammary tumor cell lines (Fig. 2 and data not shown) (Kluppel *et al.*, 1997; Quintrell *et al.*, 1987; Sato *et al.*, 1994; Umemori *et al.*, 1992; Yi *et al.*, 1991; Ziegler *et al.*, 1987). Interestingly, *Lyn* has been shown to specifically bind and phosphorylate *Tec* in hematopoietic cells *in vivo*, suggesting that *Tec* is a downstream effector of *Lyn* (Mano *et al.*, 1994; Mano *et al.*, 1996). Our finding that *Lyn* and *Tec* are also coexpressed in

mammary epithelial cells suggests that this signaling pathway may function in the mammary epithelial cells as well as in cells of hematopoietic origin.

Expression of Protein Kinases during Postnatal Mammary Development

Since the expression of regulatory molecules is frequently controlled at the level of transcription, we analyzed the temporal pattern of expression during postnatal mammary development for each of the protein kinases isolated in this screen. Kinase expression was determined in mammary glands harvested from male FVB mice and from female mice at 9 time points corresponding to developmental milestones encompassing puberty (2, 5, and 10 weeks of age), pregnancy (days 7, 14 and 20); lactation (day 9); and postlactational regression (days 2 and 7). Replicate Northern blots containing poly(A)$^+$ mRNA from each of these developmental stages were hybridized with probes prepared from catalytic domain fragments corresponding to each kinase.

As an initial control, Northern blots were hybridized with probes for the genes encoding ß-Actin, Gapdh, and cytokeratin 18 (Fig. 3). The resulting patterns were consistent with previous observations that steady-state levels of mRNA for many genes appear to decline during lactation and, to a lesser extent, late pregnancy and early postlactational regression (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Since the expression of *ß-Actin*, *Gapdh*, and cytokeratin 18 do not decrease on a per cell basis when assayed by *in situ* hybridization (J. Hartman, unpublished results), this phenomenon most likely results from a dilutional effect due to the extraordinary increase in milk protein gene expression that occurs during lactation. Expression levels for each kinase were therefore quantitated by phosphorimager analysis and normalized to *ß-actin* in order to correct for these dilutional effects.

A wide range of developmental patterns of gene expression were observed for the kinases surveyed in this study. Based on the assumption that kinases exhibiting distinctive patterns of regulation during particular stages of mammary development may be involved in regulating specific developmental events, we grouped these genes according to similarities in their developmental

expression profiles. This approach revealed an ordered set of expression profiles that suggested the coordinated regulation of protein kinases acting at different stages of mammary development. While the observation that a particular kinase is expressed in a developmentally regulated manner does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene often provide important clues to its biological function. Similarly, the identification of kinases with common developmental expression profiles may identify kinases whose developmental functions are related.

Kinase Expression during Ductal Morphogenesis

Prior to the onset of puberty at two weeks of age, the mammary glands of female FVB mice consist of a rudimentary epithelial tree originating from the nipple and penetrating a short distance into a mammary fat pad composed of fibroblasts and adipocytes. Following the onset of puberty at approximately 3 weeks of age, increased levels of ovarian steroids trigger the formation of club-shaped terminal end buds at the ends of growing mammary ducts. These are comprised of highly proliferative, relatively undifferentiated epithelial cells that give rise to the differentiated cell types of the mammary tree, and the appearance of these structures marks the onset of the rapid cellular proliferation characteristic of ductal morphogenesis. By five weeks of age, over half of the mammary fat pad is filled with epithelial ducts as a consequence of ongoing ductal elongation and branching. The completion of ductal morphogenesis occurs at 10 weeks of age when most terminal end buds have reached the edge of the fat pad and have regressed.

Several protein kinases, including c-*Abl*, *Met*, *Csk*, *Hck*, c-*Src*, *Fgfr1*, *Axl*, *Jak1*, *Jak2*, *Tyro3*, *Mlk1* and *Ctk*, exhibit similar levels of expression in the mammary glands of adult male mice and 2 week-old female mice (Figs. 4 - 9). Presumably, this reflects that fact that both adult males and prepubescent females have low levels of circulating 17ß-estradiol and possess only a rudimentary mammary epithelial tree. Conversely, several protein kinases, including *Ron, Met*, c-*Abl*, *Axl*, *Jak1*, *Tyro3*, and *Mlk1*, exhibit an increase in expression in the mammary glands of nulliparous mice between two and five weeks of age concomitant with the onset of ductal morphogenesis (Figs. 4 -

7). This pattern may reflect increases in circulating steroid hormone levels, increases in cellular proliferation, or increases in epithelial cell content (as reflected by the expression pattern of cytokeratin 18), that occur at the onset of puberty (Fig. 3). Thus, similarities and differences in hormonal environment, cellular proliferation, and epithelial content may account for changes in kinase expression patterns observed at different developmental stages.

The tyrosine kinases, *Tie1*, *Ron* and *Srm* each exhibited unique expression patterns with highest steady-state levels of mRNA occuring during the development of the virgin gland (Fig. 4 and data not shown). The endothelial-specific receptor tyrosine kinase, *Tie1*, is expressed at highest levels just prior to the onset of puberty in two week-old female mice (Fig. 4). A similar pattern of developmental expression was observed for the nonreceptor tyrosine kinase, *Srm* (data not shown). Little is currently known regarding the physiological state of the prepubescent mammary gland. However, since *Tie1* is required for establishing the structural integrity of the vascular endothelium (Sato *et al.*, 1995), this observation suggests the possibility that changes in endothelial cells or in the vasculature of the mammary gland may precede the rapid ductal growth that begins at puberty.

In contrast to *Tie1*, expression of the receptor tyrosine kinase, *Ron*, increases progressively during ductal morphogenesis, is down-regulated at the onset of pregnancy, and remains low throughout the remainder of postnatal mammary development (Fig. 4). Since the ligand for *Ron*, Macrophage Stimulating Protein, is a motility factor that promotes integrin-dependent epithelial cell migration (Wang *et al.*, 1996), it is plausible to hypothesize that *Ron* may contribute to the rapid epithelial migration characteristic of ductal morphogenesis. Consistent with this hypothesis, activation of *Ron* in epithelial cell lines results in enhanced proliferation, migration and invasion through reconstituted basement membranes (Santoro *et al.*, 1996; Tamagnone and Comoglio, 1997; Wang *et al.*, 1996). Moreover, *Ron* is overexpressed in a subset of human primary breast carcinomas (Maggiora *et al.*, 1998). Together, these observations suggest a potential role for *Ron* in epithelial invasion both during normal and neoplastic mammary development.

Kinase Expression during Pregnancy and Lactation

Early in pregnancy, alveolar epithelial cells proliferate rapidly to form alveolar buds in response to rising levels of estrogens and progesterone. Alveolar cell proliferation occurs primarily during the first half of pregnancy, whereas alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. This culminates in the withdrawal of epithelial cells from the cell cycle late in pregnancy concomitant with their terminal differentiation. Lactation, the final stage of lobuloalveolar development, occurs following parturition in the hormonal setting of high prolactin levels and declining estrogen and progesterone levels. The marked cellular changes that occur in the mammary gland during pregnancy and lactation are reflected on a molecular level by the temporally ordered expression of different milk protein genes (Robinson *et al.*, 1995). Each of the members of this class of genes undergoes a maximal increase in expression at a characteristic time during pregnancy, and can be classified as early, intermediate or late markers for mammary epithelial differentiation.

Similar to milk protein genes, clustering of protein kinases on the basis of similarities in their developmental expression patterns also yields an ordered temporal set of expression profiles throughout pregnancy and lactation (Figs. 5 and 6). Consistent with the dramatic changes that take place in the mammary gland during these developmental stages, over half of all kinases examined were regulated during lobuloalveolar development. These were grouped into two sets based on whether kinases were upregulated or downregulated during pregnancy.

Eighteen kinases were found to be upregulated during pregnancy, and examination of their temporal expression profiles indicates that successive waves of kinase expression occur at each stage of lobuloalveolar development (Fig. 5). Eleven kinases, including *EphA7*, *SLK*, c-*Abl*, *Met*, *Lyn*, *Csk*, c-*Kit* and *Egfr*, exhibited maximal upregulation during early pregnancy. A smaller number of kinases exhibited maximal upregulation during the remainder of lobuloalveolar development, including mid (*Hck*) and late (c-*Src*) stages of pregnancy, as well as lactation (c-*Akt1* and c-*Fes*).

Expression of the receptor tyrosine kinase, *EphA7*, in tissues of adult mice has principally been described in the central nervous system. In this study, we detected *EphA7* expression both in

mammary epithelial cell lines and in the mammary gland, where it is maximally upregulated during early pregnancy. Interestingly, during fetal development *EphA7* is expressed in bone marrow pro-B and pre-B cells, but not in more mature fetal B-lineage cells, or in any B-lineage cells of the adult (Aasheim *et al.*, 1997). In light of the similarities between postnatal mammary development and the embryonic development of other organs, it is possible that *EphA7* may play a lineage-specific or differentiation-dependent role in the mammary gland during early pregnancy.

The receptor tyrosine kinase, *Met*, has previously been implicated in mammary development by virtue of its ability to stimulate branching morphogenesis and lumen formation in mammary epithelial cells (Niemann *et al.*, 1998; Tsarfaty *et al.*, 1992). In addition, some studies have suggested that c-*Met* is overexpressed in a subset of human breast cancers, and the mammary glands of transgenic mice expressing the *tpr-met* oncogene develop hyperplastic alveolar nodules and carcinomas (Jin *et al.*, 1997; Liang *et al.*, 1996). Nevertheless, a role for Met in mammary development has not been directly demonstrated.

Our observation that c-*Src* is maximally upregulated during late pregnancy and lactation suggests the possibility that this kinase may promote mammary epithelial differentiation. The validity of this hypothesis is bolstered by the findings that c-*Src* activity is increased in mammary epithelial cells treated with the lactogenic hormone, prolactin, and that dominant negative forms of c-*Src* inhibit mammary epithelial differentiation *in vitro* (Sorensen and Sheffield, 1997). However, a role for c-*Src* in promoting differentiation seems inconsistent with previous studies demonstrating that c-*Src* is required for mammary tumorigenesis in transgenic mice expressing the polyomavirus middle T antigen, and that c-*Src* activity is elevated in murine mammary tumors induced by the *neu* oncogene (Guy *et al.*, 1994; Muthuswamy *et al.*, 1994). Morevover, overexpression of c-*Src* in transgenic mice results in mammary epithelial hyperplasia (Webster *et al.*, 1995). While c-*Src* may be required for certain oncogenic signaling pathways, it is also possible that the normal physiological role of this kinase during pregnancy may be to promote mammary epithelial differentiation.

Unlike other kinases analyzed in this study, both c-*Akt1* and c-*Fes* were maximally upregulated in the mammary gland during lactation. Akt1 has recently been shown to provide survival signals in response to a variety of growth factors and cytokines, and the Akt pathway is suppressed by the *PTEN* tumor suppressor gene. The further finding that germline mutations in *PTEN* predispose women to breast cancer suggests that Akt1 may be a pro-survival signal in the mammary epithelium as well (Li *et al.*, 1998; Li *et al.*, 1997; Liaw *et al.*, 1997; Stambolic *et al.*, 1998; Steck *et al.*, 1997). Consistent with this hypothesis, *Akt1* is expressed at high levels in human breast cancer cell lines. Similarly, while c-*Fes* expression has not previously been reported in the mammary gland, this kinase has been implicated in the induction of terminal myeloid differentiation, and in promoting the survival of differentiating myeloid cells (Ferrari *et al.*, 1994; Manfredini *et al.*, 1997; Yu *et al.*, 1989). The high levels of *Akt1* and c-*Fes* expression observed in the mammary gland during lactation suggest that these kinases may play a role in propagating survival signals in terminally differentiated cells. Similarly, the rapid down-regulation of *Akt1* and c-*Fes* expression at weaning may contribute to the onset of large-scale apoptosis at day 2 of postlactational involution. Such a model is consistent with the hypothesis that terminally differentiated cells are dependent on survival signals from hormones and growth factors to prevent death (Wyllie *et al.*, 1992).

Eleven protein kinases were found to be down-regulated in the mammary gland during pregnancy. In most cases, no marked changes in expression were observed until day 14 of pregnancy. However, for each kinase down-regulation persisted throughout late pregnancy, lactation and early postlactational regression in all cases (Fig. 6). The function of most of the down-regulated kinases in mammary development is unknown. The majority of down-regulated tyrosine kinases are growth factor receptors. These include *Fgfr1*, *Axl* and the insulin receptor, each of which appears to mediate mitogenic responses in mammary epithelial cells. Overexpression of *Fgfr1* and of the insulin receptor have been described in subsets of human breast cancers (Adnane *et al.*, 1991; Ugolini *et al.*, 1999; Webster *et al.*, 1996). Conversely, down-regulation of mitogenic growth factor pathways during mid and late pregnancy may be required for the withdrawal of epithelial cells from the cell cycle that accompanies terminal differentiation. This hypothesis is

consistent with the finding that down-regulation of Fgfr-mediated signaling is required for the terminal differentiation of myogenic cells during avian development (Itoh *et al.*, 1996).

Kinase Expression during Postlactational Involution

Immediately following weaning, secretory alveoli rapidly involute as the majority of mammary epithelial cells die in the apoptotic process of postlactational regression or involution. Cell death begins within two days following weaning, and by day seven of postlactational involution, intensive remodeling of the extracellular matrix has commenced.

Three protein kinases identified in this screen were found to be upregulated in the involuting mammary gland (Fig. 7). Expression of the receptor tyrosine kinase, *Tyro3*, is dramatically upregulated in the mammary gland at day 2 of involution, yet returns to pre-regression levels by day 7. Since *Tyro3* has been proposed to play a role in the growth and remodeling of the central nervous system, it is possible that it plays an analogous role in the mammary gland (Lai *et al.*, 1994; Stitt *et al.*, 1995).

Like *Tyro3*, *Mlk1* is also maximally upregulated in the mammary gland at day 2 of involution (Fig. 7). The developmental expression profiles of *Mlk1* and *Tyro3* share additional similarities as each kinase undergoes a modest initial upregulation in the mammary glands of nulliparous mice between two and five weeks of age, is further upregulated at the onset of pregnancy.

The developmental pattern of *Mlk1* expression was further investigated by *in situ* hybridization (Fig. 8). This revealed that *Mlk1* is expressed in the mammary gland in an epithelial-specific manner, as was predicted based on the similarity of its expression pattern in cell lines to that of cytokeratin 18. During puberty, *Mlk1* is preferentially expressed in epithelial cells of terminal end buds as compared to ducts (Fig. 8, cf. B and D). This finding may explain the upregulation of *Mlk1* observed in the mammary glands of five week-old mice. *In situ* hybridization further reveals that the modest upregulation of *Mlk1* expression that occurs during pregnancy is due to preferential induction of *Mlk1* expression in developing alveoli as compared to ducts, as well as confirms the dramatic upregulation of this kinase in involuting alveoli at day 2 of regression. The preferential

expression of *Mlk1* in specific structures within the epithelial compartment, such as terminal end buds and developing alveoli, demonstrates that the expression of this kinase is regulated spatially as well as temporally during mammary development.

In contrast to *Tyro3* and *Mlk1*, expression of the *Csk*-related cytoplasmic tyrosine kinase, *Ctk*, remains low throughout virgin development and pregnancy (Fig. 7). Induction of *Ctk* expression is initially observed during lactation, with maximal upregulation occuring at day 7 of involution. *Ctk* expression has previously been described only in the brain (Brinkley *et al.*, 1995). Together, the developmental patterns of expression observed for *Tyro3*, *Mlk1*, and *Ctk* suggest that these kinases may play a role in the dramatic changes that occur in the mammary gland during involution.

Proliferation-Dependent Patterns of Kinase Expression

An intriguing pattern of developmental expression was observed for the mammalian polo-like kinase, *Plk*. *Plk* expression is maximally upregulated in the mammary gland at day 7 of pregnancy, with a progressive decline in expression observed thereafter (Fig. 9). No expression was detected during lactation. Smaller increases in *Plk* expression were noted in the mammary glands of 5 week-old nulliparous animals. The observation that the upregulation of *Plk* expression coincides with peak alveolar proliferation rates during early pregnancy, as well as previous observations that *Plk* expression is cell cycle-regulated (Lee *et al.*, 1995), suggested that the developmental pattern of expression of this kinase reflects proliferative events in the mammary gland. This hypothesis is supported by the marked similarities in the expression profiles of *Plk*, cyclin A and cyclin D1 (Fig. 4). As such, these findings strongly suggest that the temporal profile of *Plk* expression reflects increases in mitotic activity that occur in the mammary gland during puberty and early pregnancy. Consistent with this hypothesis, the developmental expression of *Plk* in the mammary gland is spatially restricted to proliferating cellular compartments, particularly in terminal end buds during puberty and alveolar buds during pregnancy (data not shown).

Spatial Regulation of Kinase Expression

In addition to the diverse temporal patterns of expression observed for the kinases analyzed in this study, diverse spatial patterns of expression were also observed. Similar to *Mlk1* and *Plk*, the serine/threonine kinase, *SLK*, is upregulated in the mammary epithelium at day 7 of pregnancy (Figs. 5, 8, and 11A-D and data not shown). However, whereas *Mlk1* and *Plk* are preferentially expressed in alveoli as compared to ducts at this stage of development, *SLK* upregulation occurs in both ducts and alveoli. This observation suggests that upregulation of *SLK* expression may occur in response to signals that are distributed throughout the epithelium, rather than to changes specific to a subset of epithelial cells within a particular compartment.

Unlike the majority of kinases analyzed in this study, expression of the receptor tyrosine kinase, *Tyro10*, was restricted to the stroma of the mammary gland (Fig. 11E-H). This cell type-specificity was predicted based on our initial finding that *Tyro10* is preferentially expressed in cell lines that are negative for expression of the epithelial marker, cytokeratin 18 (Fig. 2). Within the mammary gland, *Tyro10* is expressed in regions immediately surrounding epithelial structures as well as in regions at the periphery of the mammary fat pad. Interestingly, *Tyro10* expression is strikingly heterogeneous in stromal cells, suggesting that the expression of this kinase may be restricted to a specific stromal cell type.

DISCUSSION

We have isolated 33 tyrosine kinases and 8 serine/threonine kinases that are expressed during the postnatal development of the murine mammary gland . Since transcription is one of the key steps at which gene expression is regulated, we chose to examine the mRNA expression of each of these kinases in a panel of transgenic mammary epithelial cell lines and in the mammary gland during multiple stages of development. Although protein kinases are typically regulated at the posttranslational level, the majority of kinases analyzed in this study were also found to be developmentally regulated at the mRNA level. Kinases were subsequently clustered into groups based on similarities in these expression patterns as a first step in drawing inferences about developmental processes in which they might be involved. In this manner, the panel of protein kinases identified in this study was used to produce a temporal map of developmental gene expression for the mammary gland.

While the temporal patterns of gene expression observed for the protein kinases surveyed in this study were diverse, application of a clustering approach revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. This finding suggests that a coordinated program of protein kinase expression exists that may play a role in regulating the cascade of events constituting mammary development.

A wide range of kinases was isolated in this study, including members of multiple receptor tyrosine kinase, cytoplasmic tyrosine kinase, and serine/threonine kinase subfamilies. A subset of the kinases that were identified in mammary gland samples were not expressed in the mammary epithelial cell lines tested, presumably as a result of their expression in a non-epithelial cell type. In contrast, virtually all of the kinases identified in mammary tumor cell lines were also found to be expressed in the mammary gland during development. This observation suggests that the expression of kinases detected in mammary epithelial cell lines is not merely a consequence of malignant transformation, nor of culture conditions, but rather suggests that these molecules may play a role in the normal physiology of the mammary gland.

In recent cDNA microarray experiments in the yeast, *Saccharomyces cerevisiae*, more than 60% of characterized genes that were found to be regulated in a cell cycle-dependent manner were already known to have functions related to the cell cycle (Cho *et al.*, 1998). Thus, while the finding that expression of a particular kinase is developmentally regulated does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene may provide important clues to its biological role. Kinases exhibiting distinctive patterns of regulation during mammary development may, in fact, be involved in controlling or mediating developmental events. Similarly, the identification of kinases that share developmental expression profiles may also identify kinases whose developmental functions are related.

The vast majority of protein kinases isolated in this screen currently have no recognized role in mammary development. Since poorly characterized genes whose expression fluctuate in parallel may not only be regulated by parallel pathways, but may also function in parallel pathways, RNA expression patterns may provide a straightforward means of gaining insight into roles played in mammary development. As such, extrapolation may yield insights into the role in mammary development played by kinases whose functions have been elucidated in other tissues. Conversely, insight into the function of kinases lacking previously described roles in murine development may be gained by extrapolation from patterns of kinase expression during mammary development.

The expression of a number of kinases isolated in our study has previously been reported to be restricted to tissues other than the mammary gland. Such kinases include *EphB3*, *EphA7*, *Ctk*, *Lyn*, *Hck*, and *Tec*. Each of these kinases is also expressed in the mammary epithelial cell lines tested. Our observation that the majority of these kinases are developmentally regulated in the mammary gland suggests that these molecules have additional unrecognized functions. It will be of great interest to determine whether the functions of these kinases in mammary development are analogous to their functions in other tissues.

RT-PCR screens designed to identify protein kinases expressed in mammary carcinomas or in the mammary gland have previously been reported by three groups: Lehtola et al. isolated 10 protein kinases from the human breast cancer cell line, MCF-7; Cance et al. isolated 25 protein

kinases from a human breast carcinoma and from the human breast cancer cell line, 600PEI; and Andres et al. isolated 24 protein kinases from murine mammary glands (Andres *et al.*, 1995; Cance *et al.*, 1993; Lehtola *et al.*, 1992). In total, these screens resulted in the identification of 43 protein kinases, 21 of which were also identified in the present study. In addition to these previously isolated kinases, our screen has resulted in the identification of an additional 20 kinases expressed in the mammary gland, bringing the total number of kinases identified in this manner to 63.

In aggregate, beyond providing clues to the regulation of different stages of postnatal mammary development by specific protein kinases, approaches similar to those taken here should prove useful in identifying sequences of events, as well as coherent regulatory patterns, in mammary development. Moreover, by analogy with hematopoiesis, certain kinases may be expressed in the mammary gland in a lineage-restricted manner and may thereby serve as useful markers for epithelial or stromal cell subtypes. As such, it is likely that the composite spatial and temporal pattern of kinase expression in the mammary gland at any given developmental stage can provide important information regarding its physiological state and should provide insight into the molecules that regulate different stages of postnatal mammary development. Ultimately, the finding that most kinases expressed in the mammary gland are developmentally regulated suggests that the array of kinases participating in the regulation of mammary development is considerably broader than currently appreciated.

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FIGURE LEGENDS

Table 1. Protein kinases isolated from mammary glands and mammary epithelial cell lines. Protein kinases isolated from murine mammary glands and mammary epithelial cell lines, arranged by type of kinase. The number of clones isolated for each kinase is shown to the right.

Figure 1. Amino acid sequence of novel protein kinases. Aligned amino acid sequences for isolated cDNA fragments corresponding to catalytic subdomains VIb-IX of *Bstk1*, *Bstk2* and *Bstk3*.

Figure 2. Protein kinase expression in murine breast cancer cell lines. Transformed cell lines were derived from mammary adenocarcinomas or mammary hyperplasias (HBI2) arising in transgenic animals expressing the *int-2*, c-*myc*, *neu* or v-Ha-*ras* oncogenes in the mammary gland, as indicated. Northern hybridization analysis of 6 μg of poly(A)$^+$ RNA from actively growing murine cell lines hybridized with cDNA probes specific for each of the probes indicated. Origins of the cell lines from left to right are as follows: NIH-3T3 fibroblast; non-transformed (Non-Tx): NMuMG, HC11, and CL-S1; MMTV-*int-2*: HBI2 and 1128; MMTV-c-*myc*: 8Ma1a, MBp6, M1011, M158, and 16MB9a; MMTV-*neu*: SMF, NAF, NF639, NF11005, and NK-2; MMTV-Ha-*ras*: AC816, AC711, and AC236.

Figure 3. Expression of control genes during mammary gland development. Northern hybridization analysis of mRNA expression for cytokeratin 18, Gapdh, and ß-actin, during postnatal developmental of the murine mammary gland. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. The smear of poly(A)$^+$ RNA beneath the 28S ribosomal RNA band is shown as a loading control. Note the apparent decrease in gene expression levels during lactation for all three genes, despite the similar amount of poly(A)$^+$ present. Origins of the mammary developmental time points are as follows: adult male; nulliparous females at 2 weeks

(prior to puberty), 5 weeks (during puberty), and 10 weeks (following puberty) of age; gravid females at day 7, day 14 and day 20 of pregnancy; day 9 of lactation; and day 2 and day 7 of postlactational regression.

Figure 4. Expression of protein kinases during ductal morphogenesis.

Northern hybridization analysis of *Tie1* and *Ron* expression during postnatal development of the murine mammary gland. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Figure 3. Phosphorimager analysis of Northern blots shown for Tie1 and *Ron*. Protein kinase expression was quantitated and normalized to ß-*actin* expression to correct for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels normalized to ß-actin are shown relative to adult virgin (10 wk).

Figure 5. Expression of protein kinases upregulated during pregnancy.

Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland for a selection of kinases that are upregulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Northern blots are arranged with kinases exhibiting upregulation early in pregnancy at the top, and kinases exhibiting upregulation late in pregnancy at the bottom. Origins of the mammary developmental time points are as in Figure 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to ß-actin are shown relative to adult virgin (10 wk).

Figure 6. Expression of protein kinases down-regulated during pregnancy.

Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland for a selection of kinases that are down-regulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Figure 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to ß-actin are shown relative to adult virgin (10 wk).

Figure 7. Expression of protein kinases during postlactational involution.

Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland for protein kinases that are upregulated during involution. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Figure 3. Phosphorimager analyses of Northern blots for *Tyro3* and *Ctk* are shown on the right. Expression levels normalized to ß-actin are shown relative to adult virgin (10 wk).

Figure 8. Spatial expression of *Mlk1* during mammary development.

In situ hybridization analysis of *Mlk1* expression during postnatal mammary development. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from female mice at 6 weeks of age (A-D), 16 weeks of age (E, F), day 7 of pregnancy (G, H), and day 2 of postlactational involution (I, J) hybridized with an ^{35}S-labeled *Mlk1*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Mlk1* probe. Exposure times were identical for all dark field photomicrographs to illustrate changes in *Mlk1* expression during pregnancy. al, alveoli; d, duct; lo, secretory lobule; st, adipose stroma; teb, terminal end bud. Magnification 500x.

Figure 9. Expression of protein kinases as a function of proliferation.

Northern hybridization analysis of protein kinase and cyclin expression during postnatal development of the murine mammary gland. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated timepoints was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Figure 3. Phosphorimager analysis of the Northern blot for *Plk* is shown on the right. Expression levels normalized to ß-actin are shown relative to adult virgin (10 wk).

Figure 10. Spatial expression of *SLK and Tyro3* during mammary development.

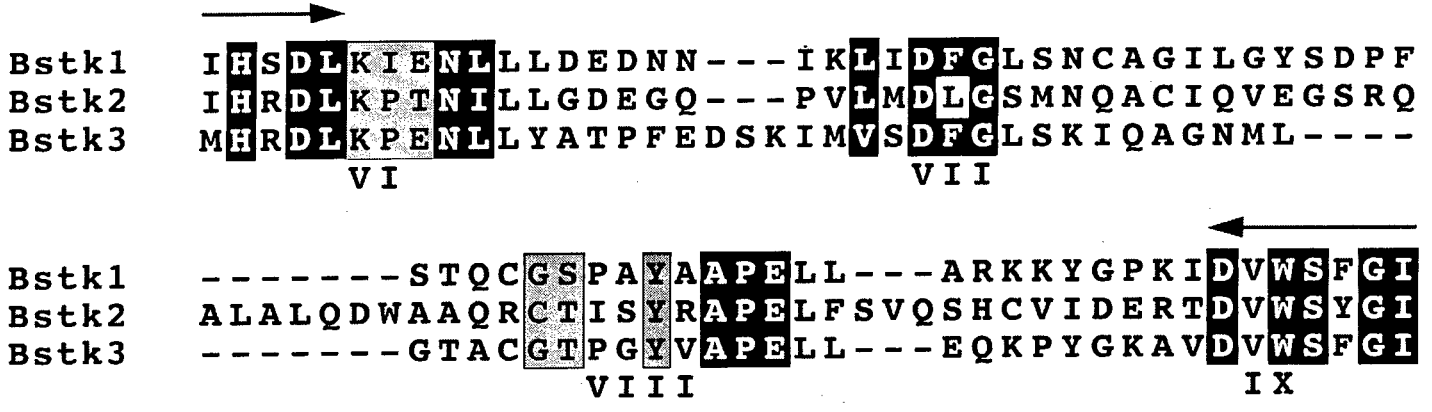
In situ hybridization analysis of *SLK* (A-D) *and Tyro3* (E-H) expression during postnatal mammary development. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from nulliparous female mice at 16 weeks of age (A-B), or day 7 of pregnancy (C-H) hybridized with ^{35}S-labeled antisense probes specific for *SLK* or *Tyro3*. No signal over background was detected in serial sections hybridized with sense *SLK* or *Tyro3* control probes. Exposure times were identical for dark-field photomicrographs for *SLK* to illustrate changes in expression during pregnancy. al, alveoli; d, duct; lu, lumen of epithelial duct; st, adipose stroma. Arrows indicate cells expressing *Tyro3*. Cells without detectable *Tyro3* expression are indicated by arrowheads. Magnification 500X.

Receptor Tyrosine Kinases	
Axl/Ufo	6
EphA2	121
EphA7	1
EphB3	2
Egfr	1
Fgfr1	126
Flt3	1
Igf1r	89
InsR	1
c-Kit	2
Met	120
MuSK	1
Neu	62
Ron	10
Ryk	1
Tie1	1
Tie2	27
Tyro10	2
Tyro3	1

Nonreceptor Tyrosine Kinases	
c-Abl	5
Csk	46
Ctk	1
c-Fes	24
Fyn	7
Hck	88
Jak1	74
Jak2	150
Lyn	21
Prkmk3	3
c-Src	23
Srm	1
Tec	1
Tyk2	4

Serine/threonine Kinases	
c-Akt1	1
Mlk1	1
Plk	26
A-Raf	1
SLK	1

Novel Kinases	
Bstk1	1
Bstk2	1
Bstk3	1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VI
VII
Bstk1 -------STQCGSPAYAAPELL---ARKKYGPKIDVWSFGI
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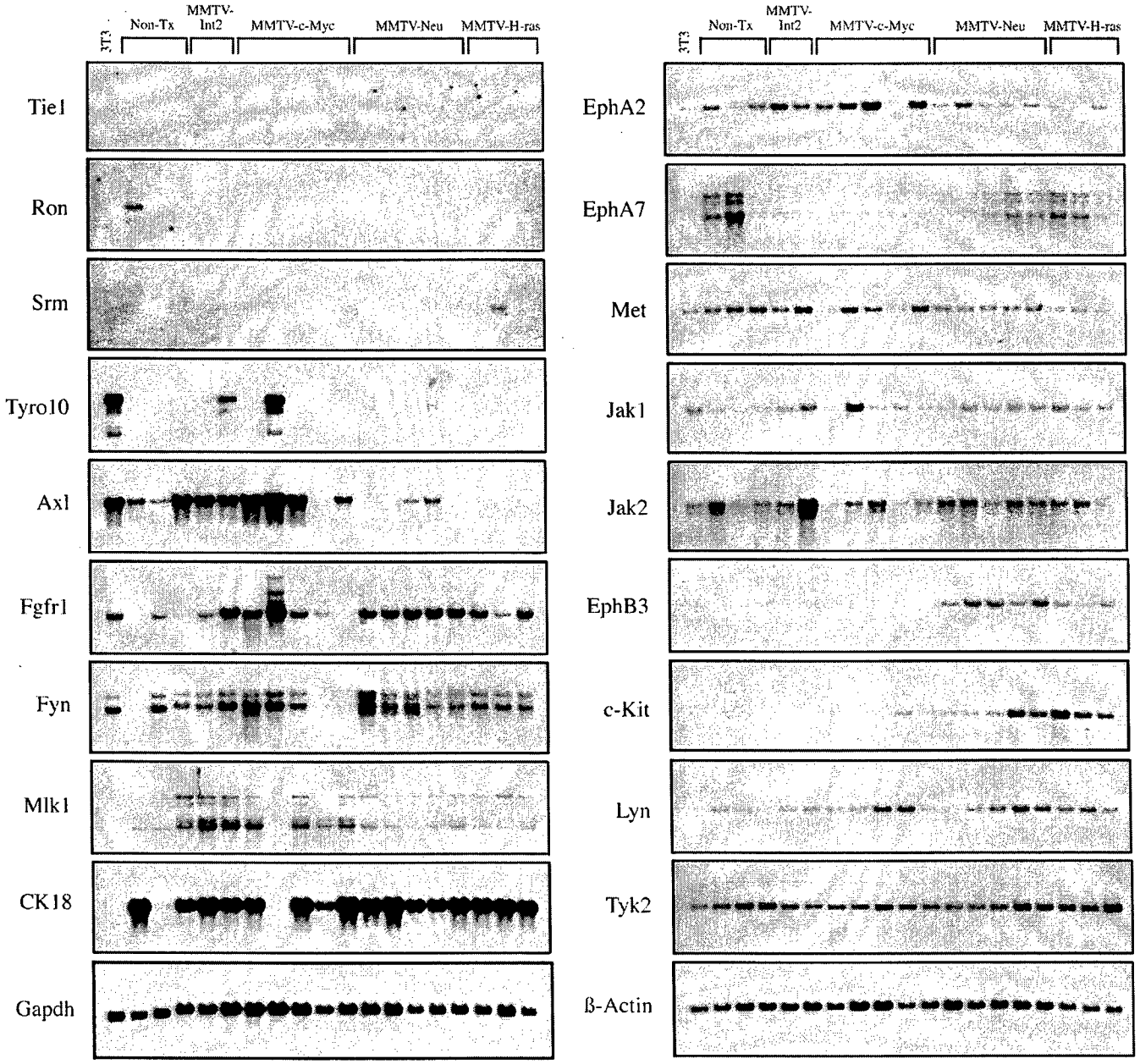
VIII
IX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MMTV-c-Myc
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Tie1
Ron
Srm
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Axl
Fgfr1
Fyn
Mlk1
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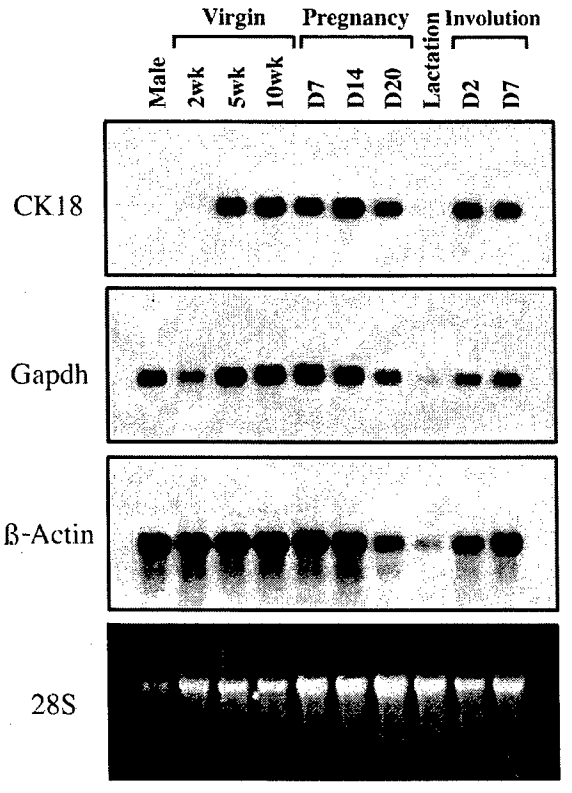
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Pregnancy
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D7
D14
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Lactation
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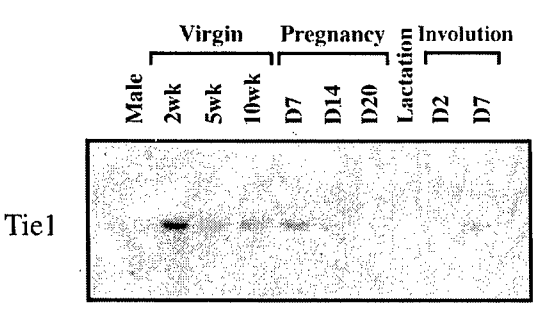
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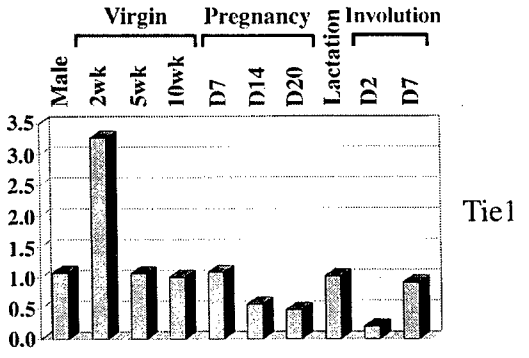
Tie1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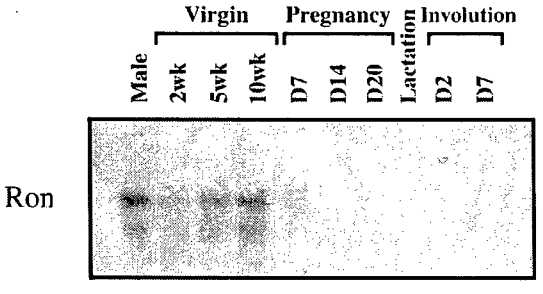
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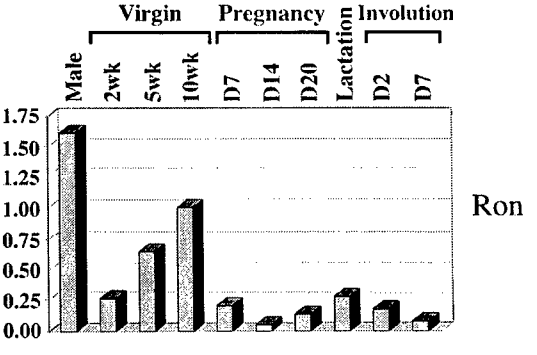
Ron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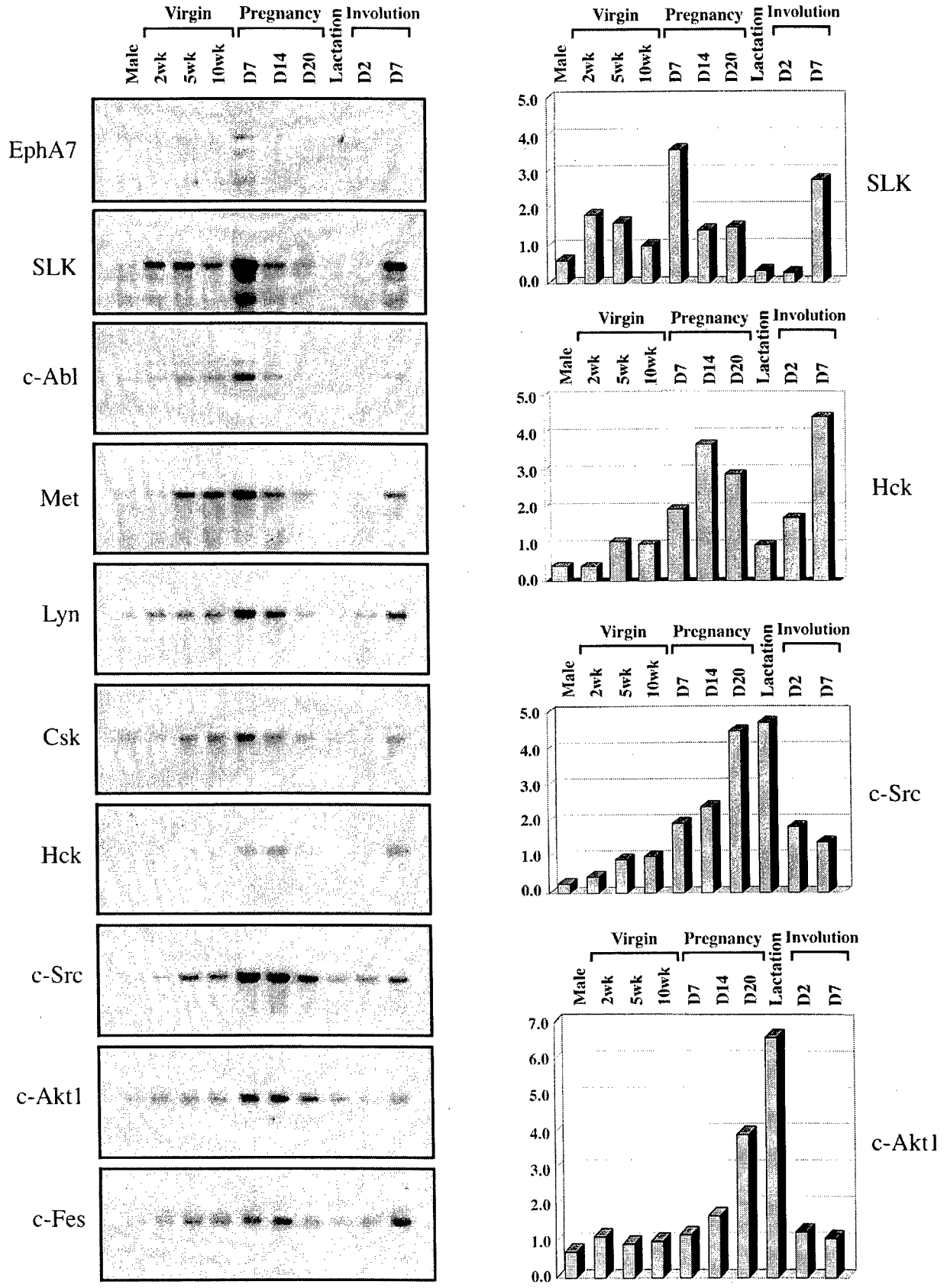
0.00
Ron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Lactation
D2
D7
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SLK
c-Abl
Met
Lyn
Csk
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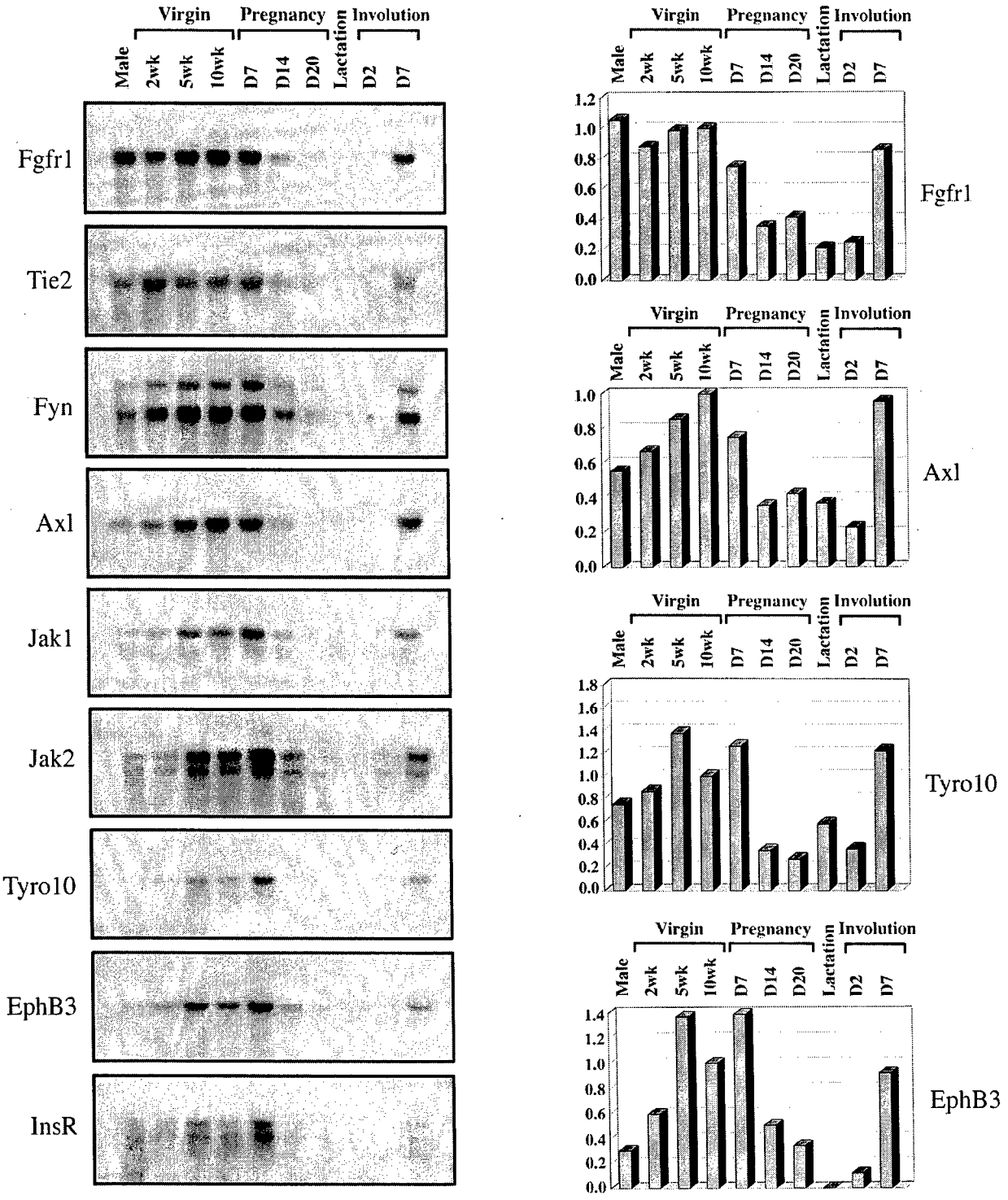
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Tie2
Fyn
Axl
Jak1
Jak2
Tyro10
EphB3
InsR
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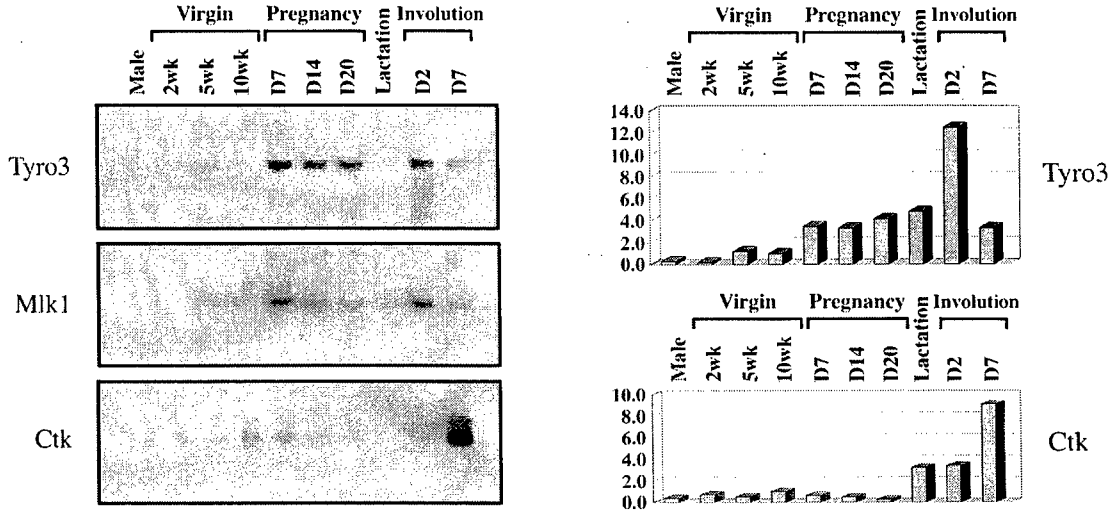
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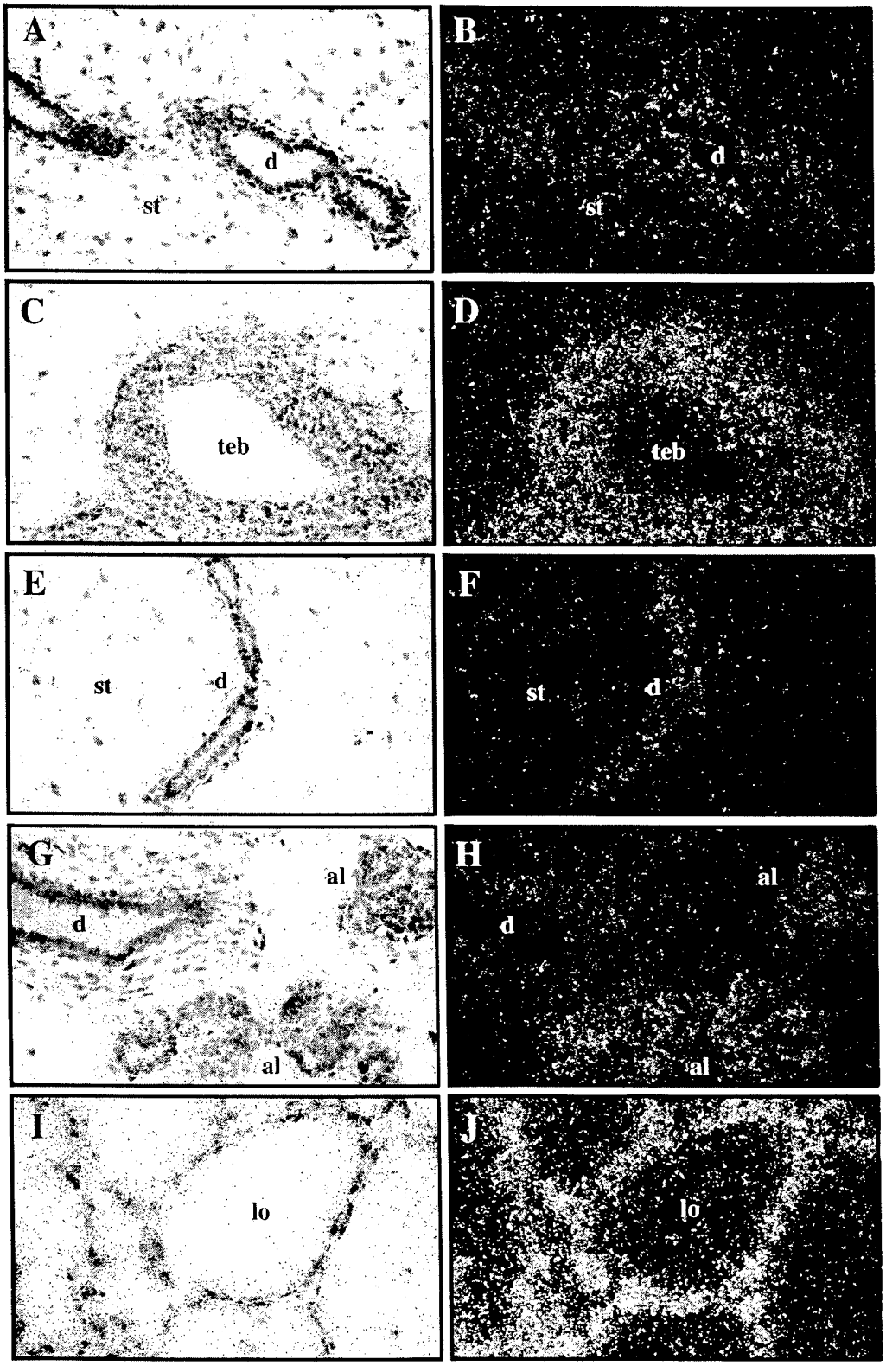
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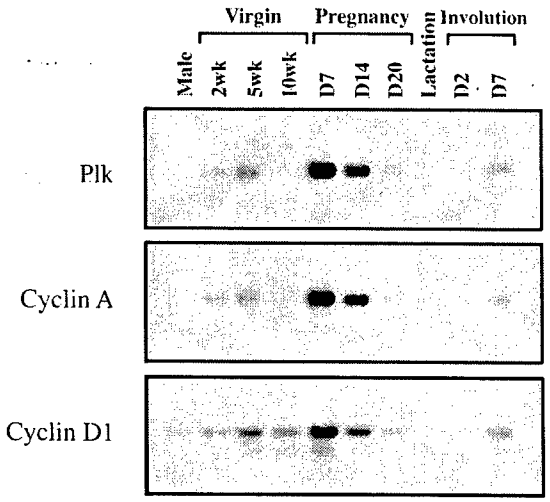
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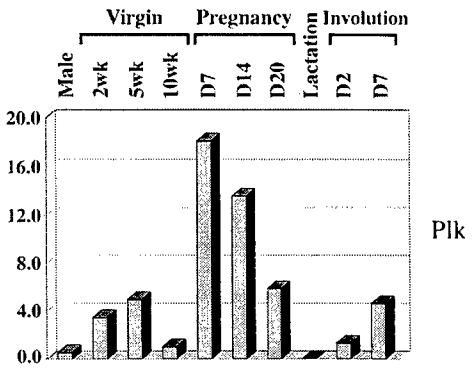
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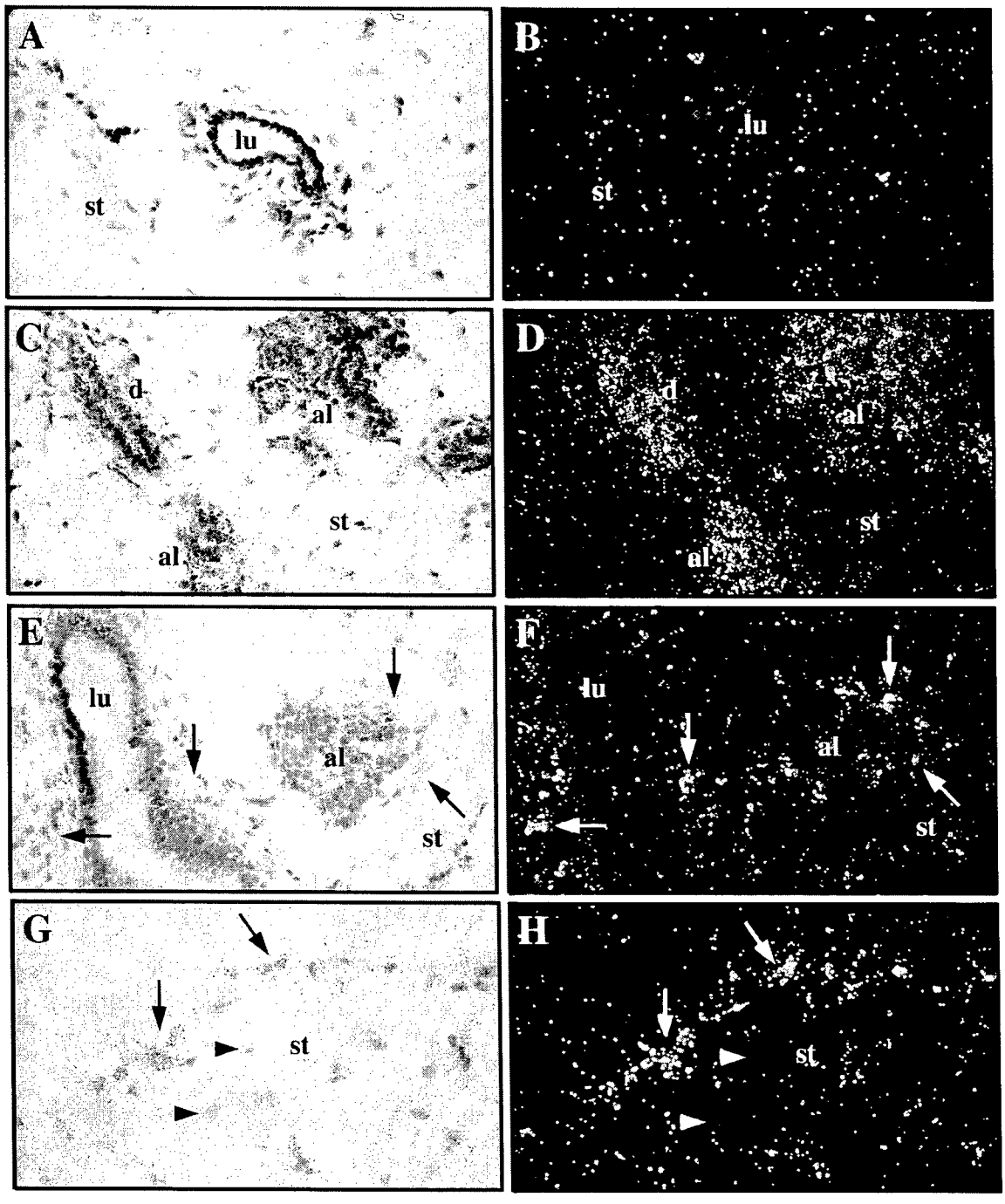
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Cloning and Characterization of *Hunk*, a Novel Mammalian SNF1-related Protein Kinase

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Running title: Cloning of a Novel Mammalian SNF1-Related Kinase

SUMMARY

We previously identified a novel protein kinase, *Hunk*, by means of a degenerate PCR screen designed to isolate kinases expressed in the murine mammary gland. We now describe the molecular cloning, chromosomal localization and activity of this kinase, and characterize its spatial and temporal pattern of expression in the mouse. We have isolated a 5.0 kb full-length cDNA clone that contains the 714 amino acid open reading frame encoding Hunk. Analysis of this cDNA reveals that Hunk is most closely related to the SNF1 family of serine/threonine kinases and contains a newly described SNF1 homology domain (SNH). Accordingly, antisera specific for Hunk detect an 80 kDa polypeptide that possesses phosphotransferase activity. *Hunk* is located on distal mouse chromosome 16 in a region syntenic to human chromosome 21q22 that is contained within the Down syndrome critical region. During fetal development and in the adult mouse *Hunk* mRNA expression is developmentally regulated and tissue-specific. Moreover, *in situ* hybridization analysis reveals that *Hunk* expression is restricted to subsets of cells within a variety of organs in the adult mouse. These findings suggest a role for Hunk in murine development.

INTRODUCTION

Major insights into the molecular mechanisms of differentiation, development, and carcinogenesis have been obtained through studies of protein kinases in a wide range of biological systems. The observation that many aberrantly regulated or aberrantly functioning protein kinases disrupt normal developmental processes or promote carcinogenesis illustrates the fact that phosphorylation events play critical roles in the regulation of cell growth and differentiation. In addition, some protein kinases are expressed in a lineage specific manner and are thereby useful markers for defining cellular subtypes (Dymecki *et al.*, 1990; Mischak *et al.*, 1991; Rawlings and Witte, 1994; Schnurch and Risau, 1993; Siliciano *et al.*, 1992; Valenzuela *et al.*, 1995).

The key role played by serine/threonine kinases in regulating diverse cellular processes is exemplified by studies of SNF1-related kinases. Several members of the SNF1 family of kinases function in signal transduction pathways involved in the cellular response to nutritional or environmental stresses (Hardie *et al.*, 1994). The *Saccharomyces cerevisiae* protein kinase SNF1, and its mammalian counterpart, AMP-activated protein kinase (AMPK), function in highly conserved signal transduction pathways that promote energy conservation. SNF1 is activated by glucose deprivation and acts by inducing the expression of genes required for growth in the absence of glucose (Celenza and Carlson, 1986; Thompson-Jaeger *et al.*, 1991). Similarly, AMPK is activated by environmental stresses that are associated with increases in the AMP:ATP ratio in the cell, such as heat shock, hypoxia, and oxidative stress (Hardie *et al.*, 1997). Once activated, AMPK functions to decrease energy-requiring anabolic pathways such as sterol and fatty acid synthesis while upregulating energy-producing catabolic pathways such as fatty acid oxidation (Moore *et al.*, 1991; Ponticos *et al.*, 1998).

In addition to their proposed role in the response to nutritional and environmental stress, SNF1 family members have also been implicated in a variety of developmental processes, including the regulation of cellular proliferation and differentiation. The murine SNF1 family member, *Msk*, is expressed in presumptive myocardial cells during embryogenesis and is downregulated following

primitive heart tube formation. This temporal pattern of expression suggests a role for *Msk* in cardiac development (Ruiz *et al.*, 1994). In an analogous manner, upregulation of the expression of the rat SNF1-related kinase, SNRK, during adipocyte differentiation suggests a role for SNRK in this process (Becker *et al.*, 1996). C-TAK1, a SNF1 kinase found in humans, has been implicated in cell cycle control by its ability to phosphorylate and inactivate Cdc25c, thereby preventing activation of Cdc2 and entry into mitosis (Peng *et al.*, 1998; Peng *et al.*, 1997). In fact, SNF1 itself has been found to mediate cell cycle arrest in response to starvation (Thompson-Jaeger *et al.*, 1991). Perhaps the most compelling evidence that SNF1 kinases are involved in development is the observation that mutations in the *C. elegans* SNF1-related kinase, PAR-1, result in an inability to establish polarity in the developing embryo (Guo and Kemphues, 1995). Similarly, asymmetric localization of the mammalian PAR-1 homologue, MARK2/Emk, in epithelial cells appears to be required for the establishment of cell polarity (Bohm *et al.*, 1997). Thus, members of the SNF1 kinase family have been demonstrated to regulate a variety of important cellular processes.

In light of the importance of protein kinases in development and carcinogenesis, we previously performed a degenerate PCR-based screen aimed at identifying protein kinases expressed in the murine mammary gland during development and in mammary epithelial cell lines derived from different transgenic mouse models of breast cancer (Chodosh *et al.*, 1999; Stairs *et al.*, 1998). In the course of these studies we identified a cDNA encoding a catalytic domain fragment from a novel protein kinase, *Hunk*. In this report, we show that *Hunk* encodes an 80 kDa polypeptide associated with phosphotransferase activity. *Hunk* is evolutionarily conserved, being most closely related to the SNF1 family of serine/threonine kinases, and is located on mouse chromosome 16 in a region syntenic to the Down syndrome critical region on human chromosome 21q22. *Hunk* expression in the mouse is developmentally regulated and tissue-specific. Interestingly, within several tissues in the adult mouse, *Hunk* is expressed in a heterogeneous pattern suggesting that the expression of this kinase is restricted to particular subtypes of cells within a variety of tissues.

MATERIALS AND METHODS

Cloning of a full-length Hunk cDNA

Poly $(A)^+$ RNA isolated from the *H-ras* transgenic mammary epithelial tumor cell line, AC816, or from FVB mouse embryos harvested at day 14 of gestation was used to generate independent cDNA libraries using either the Uni-ZAP (AC816) or Zap Express (day 14 embryo) lambda phage vector (Stratagene) according to manufacturer's instructions (Morrison and Leder, 1994). 5×10^5 plaques from each library were screened by standard methods using a [α-^{32}P] dCTP-labeled random-primed cDNA probe (BMB Random Prime). The catalytic domain fragment corresponding to nucleotides 618 to 824 of *Hunk* was used to screen two independently generated AC816 cDNA libraries. The day 14 mouse embryo cDNA library was subsequently screened using cDNA fragments corresponding to nucleotides 132 to 500 and 276 to 793 of *Hunk*. Hybridization was performed at a concentration of 10^6 CPM/ml in 48% formamide, 10% Dextran sulfate, 4.8X SSC, 20mM Tris (pH 7.5), 1X Denhart's solution, 20 µg/ml salmon sperm DNA, and 0.1% SDS at 42°C overnight. Following hybridization, blots were washed in 2 x SSC/0.1% SDS at room temperature (RT) for 30 min (x 2), followed by 0.2 x SSC/0.1% SDS at 50°C for 20 min (x 2), and subject to autoradiography (Kodak XAR-5). Positive phage clones were plaque purified and plasmids liberated by *in vivo* excision according to manufacturer's instructions (Stratagene). From each library, the independent clone with the largest insert size was completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. The full-length *Hunk* cDNA sequence has been deposited in the GenBank database (accession # AF167987).

Sequence Analysis

Sequence analysis including predicted open reading frames and calculation of predicted molecular weights was performed using MacVector (Oxford Molecular Group). Pairwise and multiple sequence alignments of kinase catalytic domains were performed using the ClustalW alignment program BLOSUM series with an open gap penalty of 10, an extend gap penalty of 0.05,

and a delay divergent of 40%. Multiple sequence alignment and phylogenetic calculations were performed using the ClustalX multisequence alignment program with the same parameters as above. DendroMaker 4.0 was used to draw an unrooted phylogenetic tree.

Tissue Preparation

FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 p.c. was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation and protein extracts were harvested from 15 to 16 week-old virgin mice and snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

Northern Analysis

RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 µl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 0.7% LE agarose gel and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1149 to 3849 of *Hunk* generated by random-primed labeling (BMB) (Marquis *et al.*, 1995). Hybridization was carried out as described above for cDNA library screening.

In Vitro Transcription/Translation

In vitro transcription/translation was performed on 1 µg of plasmid DNA using rabbit reticulocyte lysates in the presence of either ^{35}S-Met or unlabeled methionine according to manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS-PAGE gel and were subject either to autoradiography, or to immunoblotting as described below.

Generation of Anti-Hunk Antisera

GST-Hunk recombinant fusion proteins containing amino-teminal (amino acids 32-213) or carboxyl-terminal (amino acids 556-714) regions of Hunk were expressed in BL21 bacterial cells and purified using glutathione-sepharose beads according to manufacterer's instructions (Pharmacia). Following removal of the GST portion by cleavage with Prescission Protease (Pharmacia), the liberated carboxyl-terminal Hunk polypeptide was further purified by isolation on a 15% SDS-PAGE gel. Purified Hunk polypeptides either in cleavage buffer (amino-terminal) or embedded in acrylamide gel slices (carboxyl-terminal) were injected into rabbits (Cocalico Biologicals). Antisera were affinity purified on cyanogen bromide-coupled sepharose columns crosslinked with their respective antigens according to manufacturer's instructions (Pharmacia). Bound antibodies were then eluted sequentially with 100 mM glycine, pH 2.5 and 100 mM triethylamine, pH 11.5, and neutralized with 1/10 volume of 1.0 M Tris (pH 7.5) (Harlow and Lane, 1999).

Immunoblotting Analysis

Protein extracts were generated by lysing tissue culture cells or homogenizing murine mammary glands in EBC buffer composed of 50mM Tris (pH 7.9), 120mM NaCl, and 0.5% NP40 supplemented with 1 mM ß-glycerol phosphate, 50 mM NaF, 20 μg/ml aprotinin, 100 μg/ml Pefabloc (BMB), and 10 μg/ml leupeptin. Equivalent amounts of each extract were electrophoresed on 10% SDS-PAGE gels and transferred overnight onto nitrocellulose membranes. Following visualization by Ponceau staining to verify equal protein loading and even transfer, membranes were incubated with blocking solution consisting of 4% dry milk, 0.05% Tween-20 and 1x phosphate-buffed saline (PBS) at RT. Primary antibody incubation with affinity-purified antisera was performed at RT for 1 hour at a final concentration of approximately 2 μg/ml in blocking solution. Following 3 RT washes in blocking solution, blots were incubated with a 1:10,000 dilution of a horse radish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson Immuno

Research) for 30 minutes at RT. Following three washes in blocking solution and two washes 1x PBS, blots were developed using the ECL Plus system according to manufacturer's instructions (Amersham Pharmacia) followed by exposure to film (Kodak XAR-5).

Immunoprecipitation of Hunk

Protein was extracted from tissue culture cells by lysis in EBC buffer for 15 min at 4°C. From these extracts, 500 μg of protein in 250 μl of EBC was precleared with 40 μl of 1:1 protein A-Sepharose in PBS for three hours at 4°C. Precleared lysates were incubated overnight at 4°C with affinity-purified antisera raised against either the amino-terminus of Hunk (3 μg), the carboxyl-terminus of Hunk (0.1 μg), or polypeptides unrelated to Hunk (0.1 μg or 3 μg). Immune complexes were precipitated by incubating with 40 μl of 1:1 Protein A-Sepharose in PBS for 3 hr at 4°C. Complexes were washed twice with PBS, once with EBC and electrophoresed on a 10% SDS-PAGE gel. Following transfer onto nitrocellulose membranes immunoblotting was performed as described above.

Kinase Assay

Protein was extracted from snap frozen lactating murine mammary glands and from 8Ma1a cells by dounce homogenization in EBC buffer containing protease inhibitors (Morrison and Leder, 1994). Extracts containing 820 μg protein in 1 ml EBC were precleared with 40 μl 1:1 Protein A-Sepharose (Pharmacia) in PBS for 1 hr at 4°C. One quarter of the precleared lysate was incubated at 4°C overnight with 1.2 μg/ml of affinity purified antisera raised against the amino-terminus of Hunk. Immune complexes were precipitated with 40 μl 1:1 Protein A-Sepharose in phosphate buffered saline. *In vitro* kinase activity of the resulting immunoprecipitates was assayed under final reaction conditions consisting of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 100 μM dATP, 0.5 μCi/μl [γ-^{32}P] ATP, and 0.15 μg/μl Histone H1 for 45 min at 37°C. Reactions were electrophoresed on a 15% SDS-PAGE gel and were subject to autoradiography.

RNase Protection Analysis

Ribonuclease protection analysis was performed as described (Marquis *et al.*, 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 276 to 500 of the *Hunk* cDNA and 1142 to 1241 of *ß-actin* (Genbank accession # X03672) using [α-32P] UTP and the Promega *in vitro* transcription system with T7 polymerase. The ß-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA samples at 58°C overnight in 50% formamide/100 mM PIPES (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subject to autoradiography.

In Situ Hybridization

In situ hybridization was performed as described (Marquis *et al.*, 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 276 to 793 of *Hunk*. Exposure times were 6 weeks in all cases.

Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J x *M. spretus*)F_1 females and C57BL/6J males as described [20]. A total of 205 N_2 mice were used to map the *Hunk* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Hybond - N^+ nylon membrane (Amersham). A 520 bp *Eco*RI fragment corresponding to nucleotides 276 to 793 of the *Hunk* cDNA, was labeled with [α–^{32}P] dCTP using a nick translation labeling kit (Boehringer Mannheim). Washing was performed at a final stringency of 1.0 X SSCP/0.1% SDS at 65°C. A major fragment of 6.9 kb was detected in *Sac*I digested C57BL/6J DNA and a major fragment of 5.8 kb

was detected in *Sac*I digested *M. spretus* DNA. The presence or absence of the 5.8 kb *Sac*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Hunk* including *App, Tiam1,* and *Erg* has been reported previously (Fan *et al.*, 1996). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

We previously performed a screen designed to detect protein kinases expressed in the murine mammary gland and in various breast cancer cell lines in order to identify regulatory molecules potentially involved in mammary development and carcinogenesis. RT-PCR with degenerate oligonucleotide primers was employed to amplify catalytic subdomains of protein kinases expressed in 6 murine breast cancer cell lines or in the murine mammary gland during various stages of development (Chodosh *et al.*, 1999; Stairs *et al.*, 1998). Individual PCR products were subcloned and screened by a combination of DNA sequencing and colony-lift hybridization. Examination of approximately 1500 cDNA clones from this screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, three of which were novel.

One of these novel putative serine/threonine kinases, originally referred to as *Bstk1*, was first identified as a 207 bp RT-PCR product isolated from a mammary epithelial cell line derived from an adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh *et al.*, 1999). *Bstk1* was subsequently found to be upregulated in the mammary gland during early pregnancy and following treatment with estradiol and progesterone. In addition, *Bstk1* and was also found to be preferentially expressed in mammary tumor cell lines derived from MMTV-*neu* as compared with MMTV-*c-myc* transgenic mice (Gardner *et al.*, submitted). Based upon this expression pattern, *Bstk1* was renamed *Hunk*, for Hormonally-upregulated, *neu*-tumor-associated kinase.

Isolation of cDNA Clones Encoding Hunk

In order to isolate the full-length mRNA transcript from which *Bstk1* was derived, the initial 207 bp RT-PCR product was used to screen a cDNA library prepared from the transformed mammary epithelial cell line, AC816. This mammary tumor cell line was derived from an adenocarcinoma arising in an MMTV-*H-ras* transgenic mouse (Morrison and Leder, 1994). Three cDNA clones were isolated, the longest of which, clone G3, was sequenced on both strands.

Analysis of the resulting 4982 nt cDNA sequence revealed that the G3 clone encompassed the entire 207 nt sequence of *Bstk1* and possessed an open reading frame encoding the catalytic domain of a protein kinase.

Since conceptual translation of this cDNA clone revealed no in-frame stop codons upstream of the putative initiation codon a cDNA probe derived from the 5' end of G3 was used to screen a day 14 murine embryonic cDNA library. Six additional non-chimeric cDNA clones ranging in length from 4.4 to 5.0 kb were isolated from this library, each of which possessed a poly(A) tail and a restriction pattern similar to G3 (data not shown). Dideoxy sequencing of the 5' and 3' termini of these clones revealed that in all cases differences in restriction fragment lengths could be accounted for by differences in the extent of sequence at their respective 5' ends.

Clone E8, the longest cDNA clone isolated from the mouse embryonic library was completely sequenced (Fig. 1). Comparison of the 5024 nt sequence of clone E8 with that of clone G3 revealed that clone E8 contains an additional 40 nucleotides at its 5' end and that the length of a poly(T) tract in the 3' untranslated region of the two clones differs by a single nucleotide. There were no additional differences between these two clones.

The 5024 nucleotide sequence of clone E8, hereafter referred to as *Hunk*, contains the entire 207 bp RT-PCR fragment, *Bstk1,* from positions 618 to 824 (Fig. 1). *Hunk* possesses an open reading frame (ORF) 2142 nucleotides in length beginning with a putative initiation codon at nucleotide 72. Comparison of the nucleotide sequence surrounding this site with the Kozak consensus sequence, GCC$^{A}/_{G}$CCAUGG, reveals matches at positions -4, -3, and -2 (Kozak, 1987; Kozak, 1991). The nucleotide sequence of the 5' untranslated region (UTR) and the first 100 nt of the *Hunk* ORF is extremely GC-rich (>80%). Other genes bearing such GC-rich sequences have been found to be subject to translational control (Kozak, 1991). The 3'-UTR of *Hunk* is 2.8 kb in length and lacks a canonical AATAAA polyadenylation signal, and contains instead the relatively uncommon signal, AATACA, 18 nucleotides upstream of the poly(A) tract (Bishop *et al.*, 1986; Herve *et al.*, 1995; Myohanen *et al.*, 1991; Myohanen *et al.*, 1994; Parthasarathy *et al.*, 1997; Tokishita *et al.*, 1997).

While this work was in progress, a 588 nucleotide portion of the catalytic domain of *Hunk* was independently isolated by another group and shown to recognize an mRNA approximately 4 kb in length (Korobko *et al.*, 1997). Subsequently, the same group deposited a 5026 nt full-length sequence in Genbank (Accession #AF055919) which is 10 nucleotides shorter at the 5' end and 98% identical to *Hunk*. No additional information is available regarding the cloning, localization, function or *in vivo* expression of this molecule.

The conceptual ORF of *Hunk* comprises 714 amino acids and encodes a polypeptide of predicted molecular mass 79.6 kDa. This polypeptide can be divided into an amino-terminal domain of 60 amino acids, a 260 amino acid kinase catalytic domain and a 394 amino acid carboxyl-terminal domain. The carboxyl-terminal domain of Hunk contains a 46 amino acid conserved motif located 18 amino acids C-terminal to the catalytic domain that is homologous to the previously described SNF1 homology region or SNH (Becker *et al.*, 1996). The 330 amino acids carboxyl-terminal to the SNH lack homology to other known proteins.

The putative catalytic domain of Hunk contains each of the invariant amino acid motifs characteristic of all protein kinases as well as sequences specific to serine/threonine kinases (Hanks and Quinn, 1991; Hanks *et al.*, 1988). In particular, the DLKPEN motif in subdomain VIB of the *Hunk* cDNA predicts serine/threonine kinase specificity (ten Dijke *et al.*, 1994). *Hunk* also contains the serine/threonine consensus sequence, G(T/S)XX(Y/F)X, in subdomain VIII N-terminal to the APE motif conserved among all protein kinases. In addition, several amino acids in subdomains I, VII, VIII, IX, X and XI that are conserved in tyrosine kinases are absent from the *Hunk* ORF. Thus, primary sequence analysis strongly suggests that *Hunk* encodes a functional serine/threonine kinase.

In order to determine whether the length of the cDNA clone encoding Hunk is consistent with the size of the *Hunk* mRNA message, Northern hybridization was performed on poly(A)+ RNA isolated from a *Hunk*-expressing mammary epithelial cell line (Fig. 2A). This analysis revealed a predominant mRNA transcript 5.1 kb in length, as well as a less abundant transcript approximately 5.6 kb in length, suggesting that clone E8 may correspond to the shorter *Hunk* mRNA transcript.

The finding that all 6 cDNA clones isolated from a cDNA library generated from mRNA containing both 5.1 kb and 5.6 kb *Hunk* mRNA species (see below) contain poly(A) tails and are colinear, suggests that the 5.6 kb transcript may contain additional 5' or 3' sequence relative to our longest cDNA clone. The failure to identify cDNA clones containing additional 5' sequence may be related to the GC-rich nature of the 5' UTR of Hunk and the tendency of reverse transcriptase to terminate prematurely in such regions. Alternately, the difference in size between the 5.1 and 5.6 kb transcripts may be due to utilization of an alternate downstream polyadenylation site during mRNA processing.

In order to confirm the coding potential of the *Hunk* cDNA, *in vitro* transcription/translation of clone E8 was performed in the presence of ^{35}S-Met. This yielded an 80 kDa labeled polypeptide species, consistent with the 79.6 kDa predicted size of Hunk (data not shown) suggesting that the predicted initiation codon at nucleotide 72 is capable of functioning as a translation initiation site.

Detection of Hunk in Mammalian Cells

In order to detect the polypeptide encoded by the *Hunk* locus, anti-Hunk antisera were raised against recombinant proteins encoding amino teminal (amino acids 32-213) and carboxyl-terminal (amino acids 556-714) regions of Hunk. Antisera raised against the amino and carboxyl termini of Hunk each identify a polypeptide of approximately 80 kDa present in extracts from mammary epithelial cells that express *Hunk* mRNA, but not in extracts from mammary epithelial cells that do not (Fig. 2C and data not shown). In order to demonstrate that this 80 kDa polypeptide is Hunk, protein extracts prepared from two mammary epithelial cell lines that express *Hunk* mRNA and from two mammary epithelial cell lines that do not express *Hunk* mRNA were subject to immunoprecipitation/immunoblotting protocols (Fig. 2B). Immunoprecipitation of Hunk using antisera raised against the amino terminus of Hunk, followed by immunoblotting with antisera raised against the carboxyl terminus of Hunk identified an 80 kDa polypeptide only in extracts prepared from cells known to express *Hunk* mRNA (Fig. 2B). Similarly, immunoprecipitation of Hunk using antisera raised against the carboxyl terminus of Hunk, followed by immunoblotting

with antisera raised against the amino terminus of Hunk also identified an 80 kDa polypeptide only in extracts prepared from cells known to express *Hunk* mRNA (data not shown). The 80 kDa polypeptide was not detected when immunoblotting was performed on immunoprecipitates prepared from *Hunk*-expressing cells when immunoprecipitation was carried out using either of two control affinity-purified antisera (Fig. 2B and data not shown). We conclude that this 80 kDa polypeptide represents the endogenous Hunk gene product in these mammary epithelial cell lines.

In order to prove that clone E8 encodes the predominant form of Hunk found in mammary epithelial cells, we determined whether the *in vitro* translated product of clone E8 comigrates with endogenous Hunk. Immunoblotting of protein extracts prepared from the *Hunk* mRNA-expressing mammary epithelial cell line, SMF, and from rabbit reticulocyte lysates programmed with sense RNA prepared by *in vitro* transcription of clone E8, identified comigrating 80 kDa polypeptides (Fig. 2C). No band was detected in reticulocyte lysates programmed with an empty vector or in whole cell lysates from a cell line that does not express *Hunk* mRNA. The observation that the 80 kDa polypeptide identified by anti-Hunk antisera comigrates with the polypeptide obtained following *in vitro* transcription and translation of clone E8 strongly suggests that clone E8 contains the entire ORF encoding the predominant form of *Hunk* found in mammary epithelial cells. Nevertheless, due to the absence of in-frame stop codons upstream of the putative translation initiation codon, the possibility that additional 5' coding sequence exists cannot be excluded.

Predicted Structure and Homology to Previously Isolated Protein Kinases

Multiple sequence alignment was used to determine the homology between the kinase catalytic domains of Hunk and other previously isolated protein kinases (Fig. 3A). This analysis revealed that *Hunk* displays highest homology to the *Saccharomyces cerevisiae SNF1* family of serine/threonine kinases. The SNF1 family of protein kinases is comprised of at least two subfamilies. The first subfamily includes SNF1 and its plant homologues including NPK5, AKin10, BKIN12, and Rkin1 as well as the mammalian SNF1 functional homologue, AMPK (Alderson *et al.*, 1991; Carling *et al.*, 1994; Le Guen *et al.*, 1992; Muranaka *et al.*, 1994). More

recently, additional mammalian SNF1-related kinases have been identified that define a second subfamily. These include C-TAK1/p78, MARK1, MARK2/Emk, SNRK, and Msk, as well as the *C. elegans* kinase, PAR-1 (Becker *et al.*, 1996; Drewes *et al.*, 1997; Peng *et al.*, 1998; Peng *et al.*, 1997; Ruiz *et al.*, 1994). Less closely related to either subfamily are Wpk4, Melk and KIN1, SNF1-related kinases found in wheat, mice and *S. pombe*, respectively (Heyer *et al.*, 1997; Levin and Bishop, 1990; Sano and Youssefian, 1994). Similar to these more distantly related SNF1 kinases, Hunk does not appear to belong to a previously defined SNF1 subfamily. Thus, based upon homology within the kinase domain *Hunk* appears to represent a new branch of the SNF1 family tree.

Outside of a conserved kinase catalytic domain, SNF1-related protein kinases contain a region of homology referred to as the SNH or SNF1 homology domain (Becker *et al.*, 1996). Although amino acids in this motif are conserved, the functional significance of the SNH domain is unknown. Multiple sequence alignment confirms the presence of the SNH in all SNF1 family members shown in figure 3A, and permits refinement of the conserved features of this domain (Fig. 3B). This analysis reveals that the SNH is anchored approximately 20 amino acids carboxyl-terminal to the kinase domain, spans approximately 45 amino acids, and extends further towards the amino terminus than previously reported. Our consensus identifies amino acids exhibiting greater than 70% conservation among the SNF1 family members shown as well as residues that are specific for particular SNF1 kinase subfamilies.

Although most conserved residues are shared among all family members, some residues are relatively specific for a particular subfamily. For example, the consensus amino acid at position 32 of the SNH is glutamine in subfamily I SNF1 kinases and tyrosine in subfamily II kinases. Subclass-specific residues are also found at positions 37 (A *versus* V) and 45 (K/R *versus* N). More distantly related SNF1 family members such as Wpk4 and KIN1 also have SNH domains, though the degree of homology is lower and in some cases the spacing is not conserved. Outside of its kinase and SNH domains, *Hunk* displays no detectable homology to other members of the *SNF1* family or to other known molecules.

Chromosomal Localization

The mouse chromosomal location of *Hunk* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J x *Mus spretus)*F_1 X C57BL/6J] mice (Fig. 4). This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNA samples were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse *Hunk* cDNA probe. The 5.8 kb *Sac*I *M. spretus* RFLP (see Experimental Procedures) was used to follow the segregation of the *Hunk* locus in backcross mice. The mapping results indicated that *Hunk* is located in the distal region of mouse chromosome 16 linked to *App, Tiam1* and *Erg*. Although 104 mice were analyzed for every marker and are shown in the segregation analysis (Figure 4), up to 152 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci, and the most likely gene order is: centromere- *App* – 4/123 – *Hunk* – 0/130 – *Tiam1* – 4/152 - *Erg*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are – *App* – 3.3 +/- 1.6 [*Hunk, Tiam1*] – 2.6 +/- 1.3 - *Erg*. No recombinants were detected between *Hunk* and *Tiam1* in 130 animals typed in common, suggesting that the two loci are within 2.3 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 16 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *Hunk* mapped in a region of the composite map that lacks mouse mutations (data not shown).

The distal region of mouse chromosome 16 shares a region of homology with human chromosome 21q (summarized in Figure 4). In particular, *Tiam1* has been mapped to 21q22.1.

Mutations in this region of human chromosome 21 are associated with Alzheimer's disease and Down syndrome. The close linkage between *Tiam1* and *Hunk* in mouse suggests that the human homolog of *Hunk* will map to 21q22, as well. In fact, BLAST alignment of *Hunk* to sequences in Genbank reveals homology to human genomic DNA sequences cloned from 21q22.1 (gi4835629). This indicates that the human homologue of *Hunk* lies within the Down syndrome critical region believed to be responsible for many of the phenotypic features characteristic of this contiguous gene syndrome (Delabar *et al.*, 1993; Korenberg *et al.*, 1994; Rahmani *et al.*, 1989).

Hunk Encodes a Functional Protein Kinase

In order to address whether *Hunk* encodes a functional kinase, transgenic mice were engineered to overexpress Hunk in the mammary gland using the mouse mammary tumor virus LTR to direct *Hunk* expression (Gardner *et al.*, submitted). Affinity-purified amino-terminal and carboxyl-terminal anti-Hunk antisera were used in immunoblotting experiments to detect Hunk in protein extracts prepared from the mammary glands of wild-type mice or MMTV-*Hunk* transgenic mice (Fig. 5A). Consistent with the degree of overexpression estimated from steady-state mRNA levels (data not shown), substantially higher levels of Hunk were detected in extracts prepared from transgenic as compared with wild-type mammary glands. No Hunk protein was detected in extracts prepared from a mammary epithelial cell line previously shown not to express *Hunk* mRNA.

In order to demonstrate that Hunk protein levels are correlated with kinase activity, *in vitro* kinase assays were performed. Affinity-purified anti-Hunk antisera were used to immunoprecipitate Hunk from protein extracts prepared from the mammary glands of wild-type mice, transgenic mice overexpressing Hunk, or a mammary epithelial cell line known not to express *Hunk* mRNA. The resulting immunoprecipitates were incubated with [γ-^{32}P]ATP and either histone H1 or myelin basic protein as substrates (Fig. 5B and data not shown). Hunk immunoprecipitates were able to phosphorylate both histone H1 and MBP *in vitro*. As predicted based on the relative quantities of Hunk immunoprecipitated from transgenic and wild-type mammary glands, Hunk-associated phosphotransferase activity was substantially greater in

immunoprecipitates prepared from transgenic as compared to wild-type mammary glands. No activity was observed in immunoprecipitates prepared from a cell line known not to express *Hunk* mRNA. These findings demonstrate that anti-Hunk antisera co-immunoprecipitate Hunk and a phosphotransferase and strongly suggest that *Hunk* encodes a functional protein kinase.

Analysis of Hunk mRNA expression

In order to begin to analyze the biological role played by *Hunk*, the spatial and temporal pattern of mRNA expression of this gene was determined both during fetal development and in adult tissues in the mouse. Northern hybridization analysis was performed on RNA isolated from FVB embryos at embryonic days E6.5, E13.5 and E18.5 using a unique *Hunk* cDNA probe. Hunk expression was not detected at E6.5, was dramatically upregulated at E13.5, and was subsequently down-regulated at E18.5 (Fig. 6A). Similar to results obtained in mammary epithelial cells, analysis of embryonic mRNA revealed *Hunk* mRNA transcripts approximately 5.1 and 5.6 kb in length. Unlike expression in the mammary epithelial cell line, however, the 5.6 kb Hunk mRNA transcript was more abundant than the 5.1 kb transcript at E13.5, whereas the abundance of the two transcripts was equivalent at E18.5. This pattern suggests the possibility that *Hunk* transcripts are regulated in both a developmental stage-specific and tissue-specific manner.

In order to determine the spatial localization of *Hunk* mRNA expression during fetal development, ^{35}S-labeled antisense probes were used to perform *in situ* hybridization on E13.5 and E18.5 embryos (Fig. 6B-K). These studies revealed intense organ-specific expression of *Hunk* mRNA at E13.5 in the brain, skin, and developing bone, as well as more diffuse expression throughout the embryo. Expression of *Hunk* was more restricted at E18.5, with particularly prominent hybridization in the brain, lung, salivary gland, olfactory epithelium, skin, whisker hair follicles, and kidney. Thus, *Hunk* expression during fetal development occurs in a developmentally regulated and tissue-specific manner.

The distribution of *Hunk* expression in tissues of the adult mouse was analyzed by RNase protection (Fig. 7A). High levels of *Hunk* expression were detected in ovary, thymus, lung and

brain, with modest levels of expression in breast, uterus, liver, kidney and duodenum. *Hunk* mRNA expression was very low or undetectable in heart, skeletal muscle, testis, spleen and stomach.

The spatial pattern of *Hunk* expression was determined in murine tissues by *in situ* hybridization (Fig. 7B-M). Interestingly, this analysis revealed that *Hunk* is expressed in only a subset of cells within each expressing organ. In the duodenum, *Hunk* is expressed in a subset of epithelial cells located in duodenal crypts, whereas little or no expression is observed in more differentiated epithelial cells of the duodenum or in the mesenchymal compartment of this tissue (Fig. 7B, C). Heterogeneity is also observed among the crypt cells themselves whereby cells expressing *Hunk* mRNA at high levels are located adjacent to cells expressing *Hunk* at substantially lower levels. Heterogeneous expression patterns are also observed in other tissues. For instance, *Hunk* mRNA expression in the uterus is restricted to isolated epithelial cells located in mesometrial glands (Fig. 7D, E). Similarly, *Hunk* expression in the prostate is found within only a subset of ductal epithelial cells (Fig. 7F, G). *Hunk* expression in the ovary is found principally in the stroma, with little or no expression detected in developing follicles or corpora lutea (Fig. 7H, I). Hunk expression in the thymus is limited primarily to the thymic medulla with lower levels of expression in the thymic capsule (Fig. 7J, K). High-power examination revealed that, as in other tissues, expression in the thymic medulla is markedly heterogeneous (Fig. 7L). *Hunk* is expressed throughout the brain, with particularly high levels in the cortex, dentate gyrus and CA1-3 region of the hippocampus (Fig. 7M). High-power examination also revealed marked heterogeneity among different cell types in the cerebral cortex (data not shown). Thus, *Hunk* is expressed in a variety of tissues of the adult mouse, and expression within these tissues is generally restricted to a subset of cells within a particular compartment or compartments.

DISCUSSION

We initially identified the novel serine/threonine kinase, *Hunk*, in a screen designed to isolate protein kinases involved in mammary development and carcinogenesis. We have now described the cloning, chromosomal localization and activity of this kinase and have characterized its expression in the mouse. The *Hunk* locus is located on distal mouse chromosome 16, is transcribed as 5.1 kb and 5.6 kb mRNA species, and encodes an 80 kDa protein containing each of the amino acid motifs characteristic of serine/threonine kinases. Consistent with this, antisera that specifically immunoprecipitate Hunk co-immunoprecipitates phosphotransferase activity, and overexpression of Hunk in mammary epithelial cells increases the level of this phosphotransferase activity. *Hunk* expression in the mouse is developmentally regulated and tissue-specific both during fetal development and in the adult. Interestingly, within multiple tissues *Hunk* expression is restricted to small subsets of cells within specific cellular compartments. These data suggest a role for Hunk in developmental processes in multiple tissues.

Several lines of evidence suggest that the *Hunk* cDNA sequence obtained represents the full-length *Hunk* ORF. First, Northern hybridization analysis of poly(A)$^+$ RNA isolated from mammary epithelial cell lines using a *Hunk*-specific cDNA probe identifies a predominant mRNA species 5.1 kb in length, consistent with the 5025 nt cDNA sequence obtained for clone E8. Second, *in vitro* transcription/translation of clone E8 yields a polypeptide that is detected by anti-Hunk antisera that comigrates with endogenous Hunk, and whose size is consistent with that predicted for the *Hunk* ORF. Third, comparison of the sequence of clone E8 with a recently isolated human *Hunk* cDNA clone, reveals high homology within the predicted ORF and lower homology 5' of the predicted initiation codon and 3' of the predicted termination codon (H.P. Gardner *et.al.*, in preparation). Finally, the observation that anti-Hunk antisera appear to recognize a single polypeptide species in lysates from cells known to express both transcripts, argues that we have isolated the entire ORF and that the 5.6 kb *Hunk* mRNA contains additional 5' or 3'

untranslated sequence. Taken together, these observations suggest that the cDNA clones isolated represent a full-length *Hunk* transcript and contain the complete coding region.

Within the kinase catalytic domain Hunk is most closely related to the SNF1 family of protein kinases, although it appears to define a new branch on the SNF1 family tree. SNF1 is comprised of a heterotrimeric complex that is activated by glucose starvation and is required for the expression of genes in response to certain types of nutritional stress (Carlson *et al.*, 1981; Celenza *et al.*, 1989; Ciriacy, 1977; Fields and Song, 1989; Wilson *et al.*, 1996; Yang *et al.*, 1992; Yang *et al.*, 1994; Zimmermann *et al.*, 1977). Like SNF1, the mammalian SNF1-related kinase, AMPK, is involved in the cellular response to environmental stresses, particularly those which elevate cellular AMP:ATP ratios. AMPK complements the *snf1* mutation in yeast and phosphorylates some of the same targets as SNF1 (Hardie, 1999; Hardie *et al.*, 1997; Hardie *et al.*, 1999; Woods *et al.*, 1996). Like SNF1, AMPK is a heterotrimer comprised of α, ß, and γ subunits that are homologous to the subunits of SNF1 (Hardie, 1999). Thus, AMPK and SNF1 are closely related both functionally and structurally, demonstrating that the regulatory pathways in which they operate have been highly conserved during evolution.

Other SNF1 family members in plants, including Rkin1, BKIN12, AKin10, NPK5 and Wpk4 have been implicated in nutritional and environmental stress responses (Alderson *et al.*, 1991; Muranaka *et al.*, 1994; Sano and Youssefian, 1994; Wilson *et al.*, 1996) . Like Hunk, several plant SNF1 family members are expressed in a tissue-specific manner. For example, AKIN10 is expressed in roots, shoots and leaves, whereas RKIN1 is detected in developing endosperms but not in shoots (Alderson *et al.*, 1991; Le Guen *et al.*, 1992).

More recently, SNF1-related kinases have been identified in mammals and have been implicated in development processes, particularly in the regulation of cellular proliferation and differentiation. For instance, C-TAK1/p78 appears to be involved in cell cycle regulation based on its ability to phosphorylate and inactivate Cdc25c (Peng *et al.*, 1998; Peng *et al.*, 1997). Since Cdc25c controls entry into mitosis by activating Cdc2, inactivation of Cdc25c by C-TAK1 would be

predicted to negatively regulate proliferation. Consistent with this model, C-TAK1/p78 is downregulated in adenocarcinomas of the pancreas (Parsa, 1988).

PAR-1, a *C. elegans* SNF1 family member closely related to C-TAK-1/p78, is asymmetrically localized to the posterior region of the zygote and is required for establishing polarity (Guo and Kemphues, 1995). Specifically, *par-1* mutations disrupt P granule localization, asymmetric cell divisions, blastomere fates and mitotic spindle orientation during early embryogenesis. In an analogous manner, the mammalian PAR-1 homologue, MARK2/Emk, is asymmetrically localized in epithelial cells in vertebrates and expression of a dominant negative form of MARK2 disrupts both cell polarity and epithelial cell-cell contacts (Bohm *et al.*, 1997). In addition, overexpression of either MARK2 or its close family member, MARK1, results in hyperphosphorylation of microtubule-associated proteins, disruption of the microtubule array and cell death (Drewes *et al.*, 1997).

Additional SNF1-related molecules, such as Msk and SNRK, have been implicated in vertebrate differentiation and development on the basis of their temporal and spatial patterns of expression (Becker *et al.*, 1996; Ruiz *et al.*, 1994). Similarly, our analysis of *Hunk* mRNA expression patterns suggests the possibility of a developmental role for Hunk in specific tissues. *Hunk* is expressed at high levels in the embryo during mid-gestation as cells are rapidly proliferating and differentiating, and is down-regulated in the embryo prior to parturition. During fetal development, *Hunk* mRNA is expressed in a tissue-specific manner and is restricted to particular compartments within expressing tissues. Similarly, *Hunk* is expressed in a tissue-specific manner in the adult mouse and its expression is restricted to subsets of cells within these tissues. In aggregate, these data indicate that SNF1 family members participate in a wide range of developmental processes in higher eukaryotes, and suggest that Hunk may also play an important role in one or more of these processes.

Outside the catalytic domain, a region of homology exists between SNF1 family members previously described as the SNH, or SNF1 homology region (Becker *et al.*, 1996). Since the distance between the catalytic domain and the SNH also seems to be conserved and since many

kinases contain autoregulatory domains, it is plausible that the SNH domain functions to regulate kinase activity (Yokokura *et al.*, 1995). Consistent with this speculation is the presence of weak homology between the SNH domain of SNF1 kinases and the autoinhibitory domain of the closely related family of calcium-calmodulin regulated kinases (data not shown). This homology does not extend into the adjacent calmodulin binding region, consistent with the observation that SNF1 kinases are not regulated by calmodulin. Regardless, the presence of the SNH domain in all SNF1 kinases raises the possibility that members of this family of molecules may be regulated by a common mechanism.

Hunk is located on distal mouse chromosome 16 in a region syntenic to human chromosome 21q22 that is contained within the 5 Mb Down syndrome critical region that has been defined on the basis of individuals who have partial trisomy 21 (Delabar *et al.*, 1993; Korenberg *et al.*, 1994; Rahmani *et al.*, 1989). Down syndrome is the most common cause of genetic mental retardation and is associated with characteristic physical features, an increased risk of Alzheimer's disease, and developmental abnormalities of the brain, heart, gastrointestinal tract, and other organ systems. The increased expression of genes in the Down syndrome critical region is believed to be responsible for many of the physical and cognitive defects characteristic of this contiguous gene syndrome. As anticipated based on its chromosomal location, *Hunk* appears to be contained within the region of the induced mutation in Ts1Cje, a mouse model for Down syndrome containing a segmental trisomy for the region of mouse chromosome 16 homologous with the Down syndrome critical region (Sago *et al.*, 1998). In this regard, it is interesting to note that Hunk is expressed at high levels throughout the brain during murine fetal development as well as in the adult, with particularly high levels being found in the hippocampus, dentate gyrus and cortex. Whether increased Hunk expression in the brain or gastrointestinal tract during fetal development is related to the developmental abnormalities observed in these orgains in Down syndrome is unknown. Ultimately, animal models in which genes in the Down syndrome critical region are overexpressed will permit these questions to be addressed (Smith *et al.*, 1997).

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FIGURE LEGENDS

Figure 1. **Nucleotide and deduced amino acid sequence of *Hunk*.** The composite nucleic acid sequence and conceptual translation of full-length *Hunk* cDNA is shown. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in bold on the right. A light shaded box indicates the kinase catalytic domain. Dark shaded boxes denote amino acid motifs characteristic of serine/threonine kinases. The SNF1 homology region, SNH, is denoted by a hatched box. The GC-rich region in the 5'-UTR and the putative polyadenylation sequence in the 3'-UTR are underlined by thin and thick lines, respectively. An asterisk denotes the stop codon. A bracket in the 3' UTR denotes the poly(T) tract which differs in length between the two independent cDNA clones (clone E8 is shown here).

Figure 2. **Expression, identification and coding potential of *Hunk*.** A, Northern hybridization analysis of 1 μg poly(A)+ RNA from NAF mammary epithelial cells hybridized with a cDNA probe specific for Hunk. The relative migration of RNA size markers is indicated. B, Immunoprecipitation of Hunk. Antisera raised against the animo-terminus of Hunk (α-Hunk IP) or against polypeptides unrelated to Hunk (control IP) were incubated with 500 μg of protein lysates from cells that either express (+) or do not express (-) *Hunk* mRNA. Protein A-Sepharose precipitated immunocomplexes were resolved on a 10% SDS-PAGE gel, transferred onto nitrocellulose, and immunoblotted with antisera raised against the carboxyl-terminus of Hunk. C, Immunoblotting analysis of Hunk protein using antisera raised against the carboxyl-terminus of Hunk. IVT reactions were performed in rabbit reticulocyte lysates in the presence of unlabeled methionine using 1 μg of either plasmid control (vector) or full-length Hunk cDNA (E8) as a template. Equivalent amounts of these IVT reactions were resolved on a 10% SDS-PGE gel along with lysates containing 275 μg of protein from *Hunk*-expressing (+) and non-expressing (-) cell lines used as controls for the relative migration of endogenous Hunk. Note that the *in vitro*

translated product detected with anti-Hunk antisera comigrates with the endogenous form of Hunk protein. The relative migration of the closest molecular weight marker is indicated.

Figure 3. **Hunk represents a member of a novel subfamily of SNF1-related serine/threonine kinases.** A, Phylogenetic tree illustrating the relationship of Hunk kinase catalytic subdomains I-XI to other SNF1 family members. Analysis and depiction of results was performed using the ClustalX multisequence alignment program and DendroMaker 4.0. B, Amino acid alignment of SNF1 family members demonstrating conserved residues in the SNF homology domain. Positions at which an amino acid occurs with greater than 70% frequency are indicated in bold with dark shading. Positions at which similar amino acids occur with greater than 70% frequency are shown with light shading. A consensus sequence for all conserved residues is shown in bold at the bottom. Residues conserved within subfamilies are shown on the consensus line unbolded and separated by a gray line. A gray line also separates members of the two SNF1 subfamilies as denoted on the left side. Gaps (-) were introduced to maximize the alignment. Numbering is shown on top and is relative to Hunk spacing. Database accession numbers used are: 80944 (Wpk4); 3089349 (C-TAK1); Z83868 (MARK1); Z83869 (MARK2); U22183 (PAR-1); U11494 (Msk); X89383 (SNRK); JC1446 (AKin10); A56009 (NPK5); S24578 (BKIN12); A41361 (Rkin1); Z29486 (AMPK); A26030 (SNF1); X95351 (Melk); and A38903 (*S. pombe* KIN1).

Figure 4. ***Hunk* maps in the distal region of mouse chromosome 16.** *Hunk* was mapped to mouse chromosome 16 by interspecific backcross analysis. The segregation patterns of *Hunk* and flanking genes in 104 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 104 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J x *M. spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring

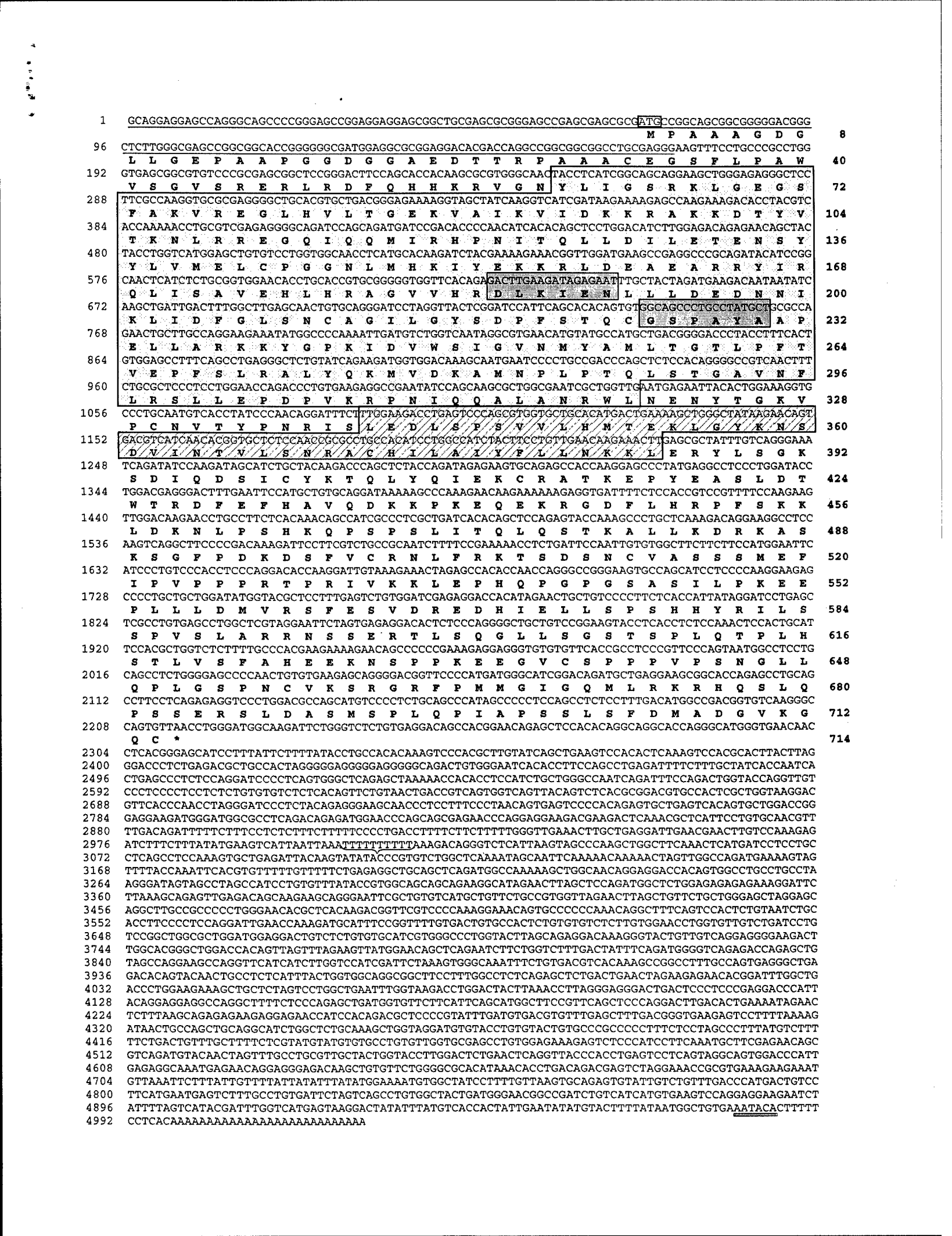
inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 16 linkage map showing the location of *Hunk* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

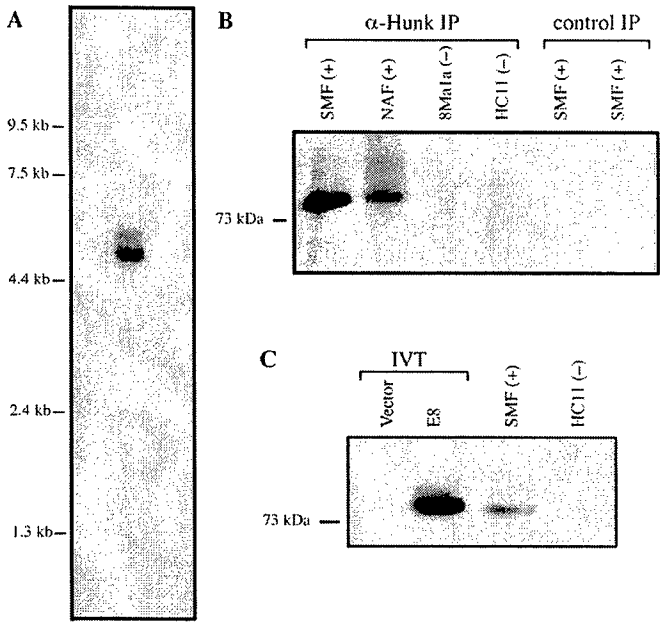
Figure 5. ***Hunk* encodes a functional protein kinase.** A, Immunoblotting using amino terminal anti-Hunk antisera to analyze Hunk protein expression. 50 μg of protein extract prepared from mammary glands harvested from either MMTV-Hunk transgenic (TG) or wild-type (WT) mice, or 100 μg of protein extract prepared from HC11 cells, a mammary epithelial cell line known not to express *Hunk* mRNA (-), were resolved on a 10% SDS-PAGE gel and analyzed by immunoblotting using amino terminal anti-Hunk antisera. The relative migration of the 78 kDa molecular weight marker is indicated. B, *In vitro* kinase assay of Hunk immunoprecipitates. Histone H1 was used as an *in vitro* kinase substrate for Hunk protein immunoprecipitated from extracts containing 205 μg of protein as in Figure 2B. Immunoprecipitates were incubated with [γ-32P] ATP and histone H1, separated on a 10% SDS-PAGE gel and subject to autoradiography. An arrowhead indicates the relative migration of histone H1.

Figure 6. **Expression of *Hunk* during murine embryogenesis.** A, Northern hybridization analysis of 2 μg of poly(A)+ RNA from day E6.5, E13.5, and E18.5 embryos hybridized with a cDNA probe specific for *Hunk*. The 28S ribosomal RNA band is shown as a loading control. B-K, *In situ* hybridization analysis of *Hunk* mRNA expression . Bright-field (D, F, G, H) and dark-field (B, C, E, I, J, K) photomicrographs of E13.5 (B) and E18.5 (C-K) FVB embryo sections hybridized with an ^{35}S-lableled *Hunk* antisense cDNA probe. Tissues shown are kidney (D, E), whisker hair follicles (F, I), submandibular gland (G, J), and skin (H, K). No signal over

background was detected in serial sections hybridized with sense *Hunk* probes. bo, bowel; fv, fourth ventricle; ki, kidney; li, liver; lu, lung; lv, lateral ventricle; oe, olfactory epithelium; sg, submandibular gland; sk, skin; st, stomach; wf, whisker hair follicle. Magnification: 8X (B, C); 20X (D-K). Exposure times were optimized for each panel.

Figure 7. **Tissue-specific expression of *Hunk* in adult tissues.** A, RNase protection analysis of *Hunk* mRNA expression in tissues of the adult mouse. 30 μg of RNA isolated from the indicated murine tissues was hybridized with antisense RNA probes specific for *Hunk* and for ß-actin, digested with RNase A and T1, and resolved by electrophoresis on a 6% denaturing polyacrylamide gel, dried and subjected to autoradiography. B-M, Spatial localization of *Hunk* expression in tissues of the adult mouse. Bright-field (B, D, F, H, J) and dark-field (C, E, G, I, K, L, M) photomicrographs of *in situ* hybridization analysis performed on sections of duodenum (B, C), uterus (D, E), prostate (F, G), ovary (H, I), thymus (J-L), and brain (M), hybridized with an ^{35}S-labeled *Hunk* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Arrows indicate cells expressing *Hunk* at high levels. CA1 and CA3, regions of the hippocampus; cl, corpus luteum; co, cortex; d, epithelial duct; dg, dentate gyrus; eg, endometrial gland; fo, follicle; ic, intestinal crypt; me, medulla; mg, mesometrial gland; mu, mucosa; pc, parietal cortex; se, serosa; st, stroma. Magnification: 10X (M), 90X (H-K), 180X (B, C), 300X (D, E) or 500X (F, G, L, M).

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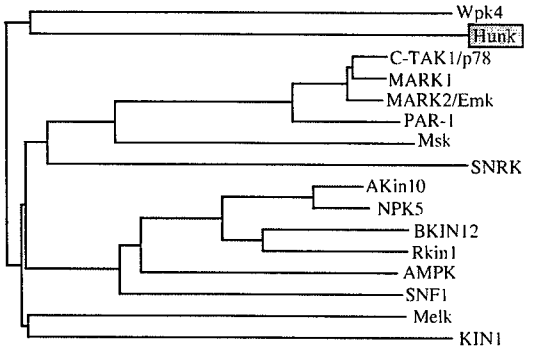
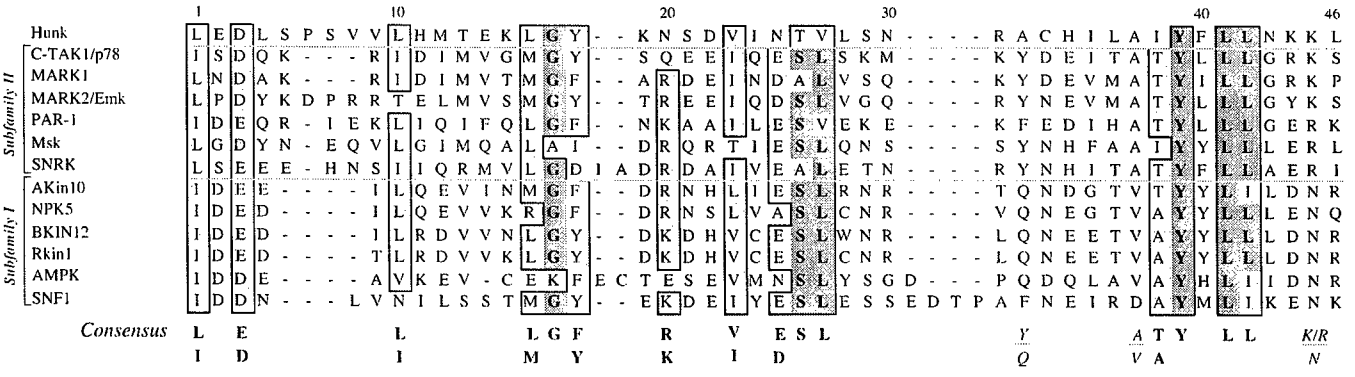


B


1
10
20
30
40
46
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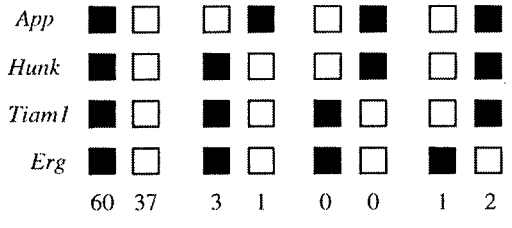
C-TAK1/p78 I S D Q K - - - R I D I M V G M G Y - - S Q E E I Q E S L S K M - - - - K Y D E I T A T Y L L L G R K S
MARK1 L N D A K - - - R I D I M V T M G F - - A R D E I N D A L V S Q - - - - K Y D E V M A T Y I L L G R K P
MARK2/Emk L P D Y K D P R R T E L M V S M G Y - - T R E E I Q D S L V G Q - - - - R Y N E V M A T Y L L L G Y K S
PAR-1 I D E Q R - I E K L I Q I F Q L G F - - N K A A I L E S V E K E - - - - K F E D I H A T Y L L L G E R K
Msk L G D Y N - E Q V L G I M Q A L A I - - D R Q R T I E S L Q N S - - - - S Y N H F A A I Y Y L L L E R L
SNRK L S E E E - H N S I I Q R M V L G D I A D R D A I V E A L E T N - - - - R Y N H I T A T Y F L L A E R I
AKin10 I D E E - - - - I L Q E V I N M G F - - D R N H L I E S L R N R - - - - T Q N D G T V T Y Y L I L D N R
NPK5 I D E D - - - - I L Q E V V K R G F - - D R N S L V A S L C N R - - - - V Q N E G T V A Y Y L L L E N Q
BKIN12 I D E D - - - - I L R D V V N L G Y - - D K D H V C E S L W N R - - - - L Q N E E T V A Y Y L L L D N R
Rkin1 I D E D - - - - T L R D V V K L G Y - - D K D H V C E S L C N R - - - - L Q N E E T V A Y Y L L L D N R
AMPK I D D E - - - - A V K E V - C E K F E C T E S E V M N S L Y S G D - - - P Q D Q L A V A Y H L I I D N R
SNF1 I D D N - - - L V N I L S S T M G Y - - E K D E I Y E S L E S S E D T P A F N E I R D A Y M L I K E N K
Subfamily II
Subfamily I
Consensus L E L L G F R V E S L Y A T Y L L K/R
I D I M Y K I D Q V A N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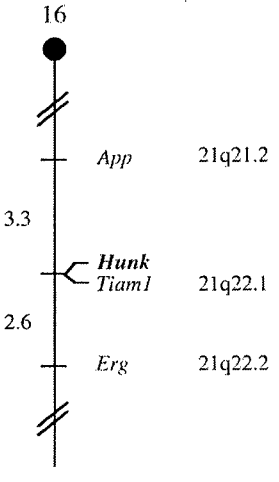
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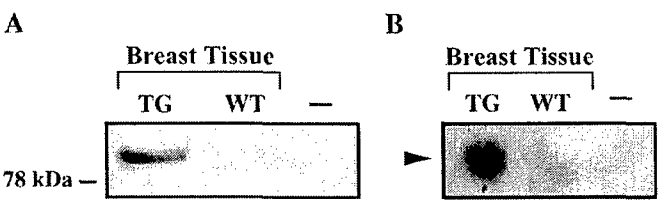
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WT
—
78 kDa —
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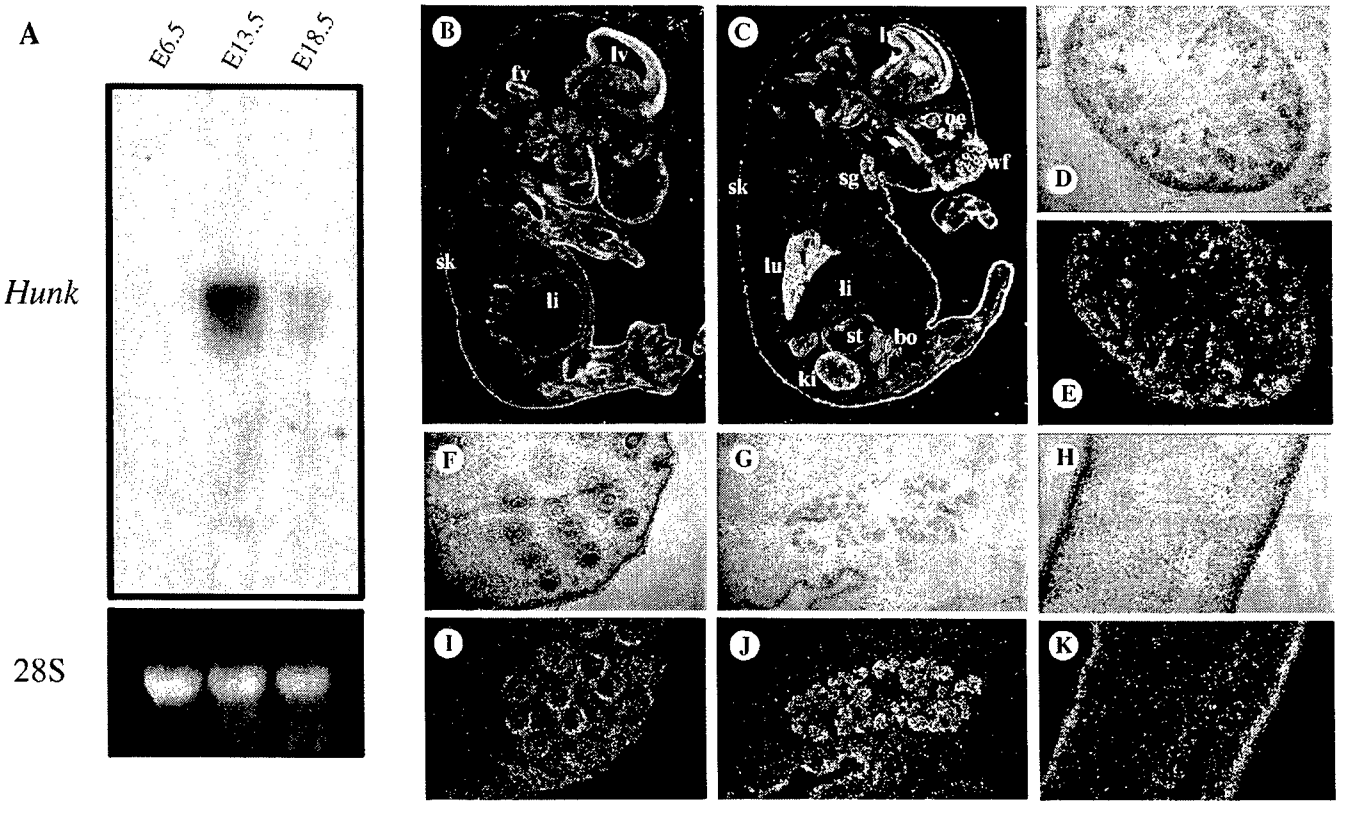
TG
WT
—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E18.5
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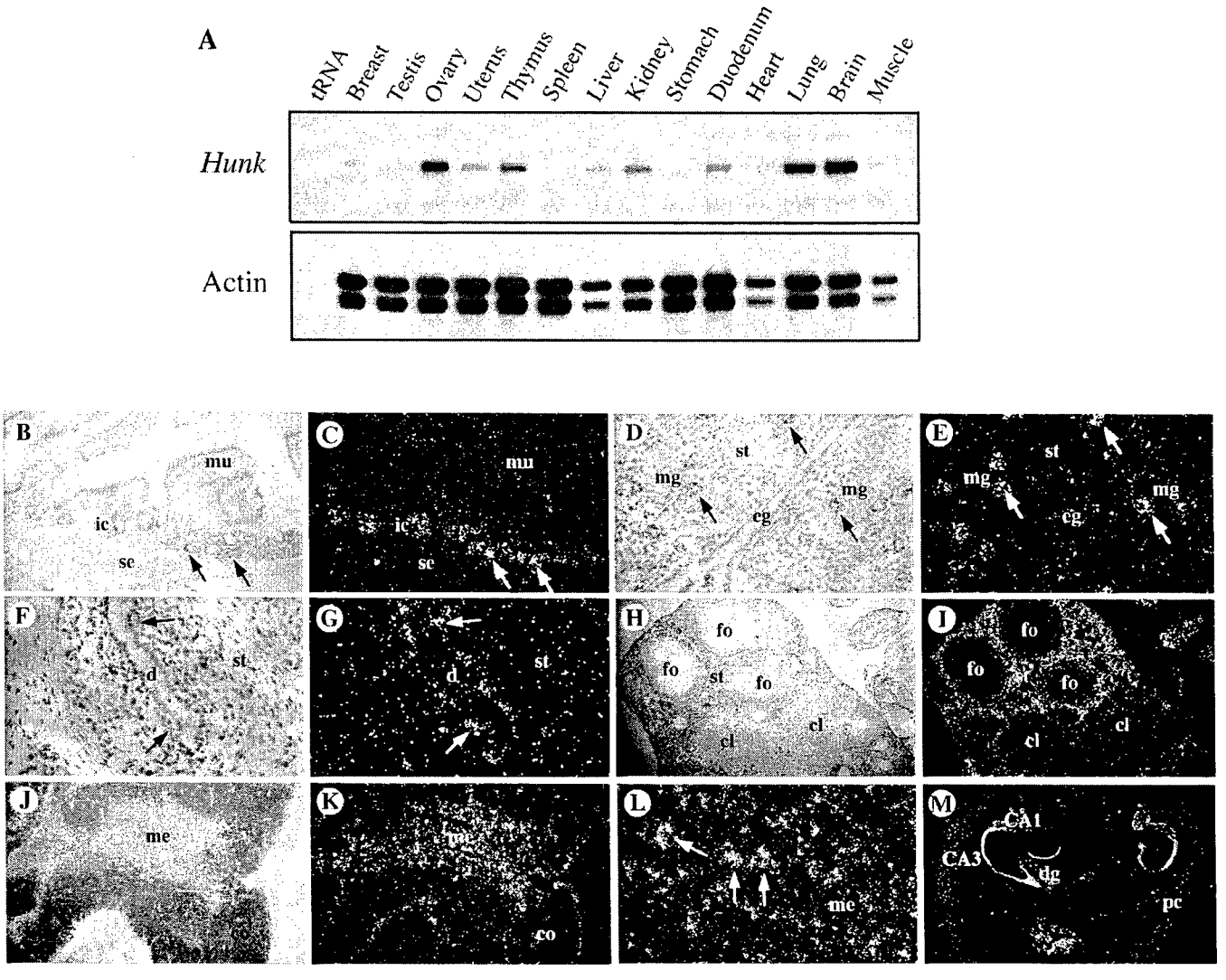
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Thymus
Spleen
Liver
Kidney
Stomach
Duodenum
Heart
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Muscle
Hunk
Actin
B
mu
ic
se
C
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Cloning, Characterization, and Chromosomal Localization of *Punc*, a Calcium/Calmodulin-Dependent Protein Kinase

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Running title: Cloning of the CaM Kinase, *Punc*

Genbank accession # AF181984

Abstract

Calcium is an important second messenger in eukaryotic cells. Many of the effects of calcium are mediated via its interaction with calmodulin and the subsequent activation of Ca^{2+}/calmodulin-dependent (CaM) kinases. CaM kinases are involved in a wide variety of cellular processes including muscle contraction, neurotransmitter release, cell cycle control and transcriptional regulation. While CaMKII has been implicated in learning and memory, the biological role of other multifunctional CaM kinases, CaMKI and CaMKIV, are largely unknown. In the course of a degenerate RT-PCR protein kinase screen, we identified a novel serine/threonine kinase, *Punc*. In this report, we describe the cloning, chromosomal localization and expression of *Punc*, which encodes a 38 kDa protein kinase whose catalytic domain shares 45-70% identity with members of the CaM kinase family. The gene for *Punc* localizes to mouse chromosome X, in a region that is syntenic with human chromosome Xq28 and associated with multiple distinct mental retardation syndromes. *Punc* is upregulated during intermediate and late stages of murine fetal development with highest levels of expression in developing brain, bone, and gut. *Punc* is also expressed in a tissue-specific manner in adult mice with highest levels of expression detected in brain, uterus, ovary, and testis. Interestingly, *Punc* expression in these tissues is restricted to particular compartments and appears to be further restricted to subsets of cells within those compartments. The chromosomal localization of *Punc*, along with its tissue-specific and restricted pattern of spatial expression during development suggest that *Punc* may be involved in a variety of developmental processes, including that of the central nervous system.

Introduction

Studies of protein kinases in a wide range of biological systems have led to a more comprehensive understanding of the regulation of cell growth and differentiation (Bolen, 1993; Fantl, 1993; Hardie, 1990). Accordingly, many protein kinases function as intermediates in signal transduction pathways that control complex processes such as differentiation, development and carcinogenesis (Birchmeier*et al.*, 1993; Bolen*et al.*, 1992; Rawlings and Witte, 1994). Not surprisingly, several members of the protein kinase family have been shown to be involved in the pathogenesis of breast cancer both in humans and in rodent model systems (Cardiff and Muller, 1993; Dickson*et al.*, 1992; Guy*et al.*, 1994; Guy*et al.*, 1992; Slamon*et al.*, 1989). In light of these findings, we performed a screen designed to identify and study the role of protein kinases in mammary development and carcinogenesis (Chodosh*et al.*, 1999; Gardner*et al.*, Submitted; Stairs*et al.*, 1998). In the course of these studies we identified a novel serine/threonine kinase, *Punc*, (Pregnancy upregulated non-ubiquitously expressed CaM kinase), that is related to the Ca^{2+}/calmodulin-dependent (CaM) family of protein kinases.

Calcium is an important intracellular second messenger molecule in eukaryotic signal transduction pathways. Many of the effects of calcium are mediated through its interaction with the calcium binding protein, calmodulin. The Ca^{2+}/calmodulin complex is, in turn, required for maximal activation of CaM-dependent protein kinases, which ultimately regulate cellular processes as diverse as neurotransmitter release, metabolism, and gene transcription (Fukunaga and Miyamoto, 1999; Lukas*et al.*, 1998; Matthews*et al.*, 1994; Nairn and Picciotto, 1994; Polishchuk*et al.*, 1995; Schulman, 1993; Sheng*et al.*, 1991). In addition to their regulation by CaM, CaM kinases share structural and functional homology both in the kinase catalytic domain and in a regulatory region comprised of composite autoinhibitory and CaM-binding domains (Hanks and Quinn, 1991; Hanks*et al.*, 1988; Haribabu*et al.*, 1995; Knighton*et al.*, 1992; Picciotto*et al.*, 1996; Yokokura*et al.*, 1995),

Despite these similarities, significant differences exist between CaM kinase family members. For instance, this family includes members with high substrate specificity, such as

myosin light-chain kinase (MLCK) and phosphorylase kinase, as well as members with broader substrate-specificities such as CaMKI, CaMKIV and members of the CaMKII subfamily that are collectively referred to as the multifunctional CaM kinases (Braun and Schulman, 1995; Cawley*et al.*, 1993; Herring*et al.*, 1990; Matthews, *et al.*, 1994; Schulman, 1993). Other properties that differ among CaM kinase family members include their subcellular localization, regulation by autophosphorylation, and regulation by other proteins. In addition, CaM kinases have unique amino- and carboxyl- terminal domains that contribute to kinase-specific differences in subcellular localization, subunit interactions, and other protein-protein interactions.

Much of the information available regarding the multifunctional CaM kinases is derived from studies conducted in the brain, in part because each of these kinases is expressed at high levels in this organ. However, CaMKII is the only multifunctional CaM kinase with a defined biological role (Hanley*et al.*, 1987; Jensen*et al.*, 1991b; Lin*et al.*, 1987; Picciotto*et al.*, 1993; Tobimatsu and Fujisawa, 1989; Tobimatsu*et al.*, 1988). The CaMKII holoenzyme is an oligomeric complex comprised of combinations of independently encoded, highly homologous α, β, γ, and δ subunits. Mice with targeted disruption of CaMKIIα are deficient in long term potentiation and exhibit specific defects in learning and memory (Silva*et al.*, 1992a; Silva*et al.*, 1992b). Unlike the α subunit of the CaMKII whose expression is restricted to the brain, CaMKI and CaMKIV as well as the δ and γ subunits of CaMKII have a broader tissue distribution, and therefore presumably have as yet unrecognized functions in other tissues (Hanley, *et al.*, 1987; Lin, *et al.*, 1987; Naito*et al.*, 1997; Picciotto, *et al.*, 1993; Picciotto*et al.*, 1995; Tobimatsu and Fujisawa, 1989; Tobimatsu, *et al.*, 1988).

In this report, we describe the cloning, chromosomal localization and initial characterization of *Punc*, a member of the CaM kinase family of protein serine/threonine kinases. We have isolated cDNA clones for *Punc* that encode a 38 kDa polypeptide. The gene for *Punc* localizes to mouse chromosome X in a region syntenic with human chromosome Xq28 that has been implicated in distinct mental retardation syndromes (Lubs*et al.*, 1999). *Punc* expression is developmentally regulated and tissue-specific during murine fetal development with high levels of expression in

developing brain, bone, and gut. *Punc* expression is also tissue-specific in adult mice with highest levels of expression in the hippocampus and dentate gyrus of the brain. Interestingly, within expressing tissues, *Punc* expression is restricted to subsets of cells within particular compartments. These data suggest a role for *Punc* in the development of the central nervous system and other tissues.

Materials and Methods

Cloning of a full-length Punc cDNA

The original catalytic domain fragment, *Bstk3*, corresponding to nucleotides 501 to 704 of full-length *Punc* was used to screen 5 X 10^5 lambda phage plaques from an oligo(dT)-primed murine brain cDNA library according to standard protocols (CPMB). Primary screening yielded a total of 73 clones of varying hybridization intensity that were positive on duplicate filters. Ten clones with medium to high hybridization intensity were plaque purified and plasmids liberated by *in vivo* excision according to manufacturer's instructions (Stratagene). Sequence analysis of five of these clones revealed high homology to CaMKI. The remaining five clones were found to encode portions of *Punc* as determined by overlapping sequence identity to each other or to *Bstk3*. Two clones were not studied further since one clone was chimeric and a second clone contained only partial *Punc* sequence. Three non-chimeric clones, U7, V1 and Q3, were completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. Nucleotide sequence alignment revealed no differences between the three clones outside of their respective 5'-UTR sequences. The full-length *Punc* cDNA sequence corresponding to the clone with the longest 5'-UTR, U7, has been deposited in the GenBank database (accession # AF181984).

Sequence Analysis

Sequence analysis, including prediction of open reading frames, calculation of predicted molecular weights, multiple sequence alignment and phylogenetic analysis, was performed using MacVector (Oxford Molecular Group), ClustalW, ClustalX and DendroMaker 4.0. Pairwise and multiple sequence alignments of kinase catalytic domains I-XI were performed using the ClustalW alignment program. Calculations were made using the BLOSUM series with an open gap penalty of 10, an extend gap penalty of 0.05, and a delay divergent of 40%. Phylogenetic calculations with the same parameters were performed using the ClustalX multisequence alignment program. An unrooted phylogenetic tree was drawn using DendroMaker 4.0.

Tissue Preparation

FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 p.c. was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation and protein extracts were harvested from 15 to 16 week-old virgin mice and snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

RNA Analysis

RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 µl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis*et al.*, 1995; Rajan*et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1% LE agarose gel and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1135 to 1509 of *Punc* generated by random-primed labeling (BMB) (Marquis, *et al.*, 1995)

Southern hybridization analysis was performed on a zooblot (Clontech) hybridized with a ^{32}P-labeled cDNA probe corresponding nucleotides 1321 to 1509 from the 3'-UTR of *Punc*. Hybridization and washes were performed according to manufacturer's directions (Clontech). A single band was detected in genomic DNA from both mouse and rat confirming that, under these conditions, a single locus is recognized by this *Punc*-specific 3'-UTR probe.

Ribonuclease protection analysis was performed as described (Marquis, *et al.*, 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 1321 to 1509 of *Punc* and 1142 to 1241 of *ß-actin* (Genbank accession # X03672) using [α-32P] UTP and the Promega *in vitro* transcription system with T7 polymerase. A ß-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA

samples at 58°C overnight in 50% formamide/100 mM PIPES (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subject to autoradiography.

In Vitro Transcription/Translation

In vitro transcription/translation was performed on 1 µg of plasmid DNA using rabbit reticulocyte lysates in the presence of ^{35}S-methionine according to manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS-PAGE gel and were subject to autoradiography.

Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J x *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Punc* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins*et al.*, 1982). All blots were prepared with Hybond - N^+ nylon membrane (Amersham). The probe, a 375 bp fragment corresponding to nucleotides 1135 to 1509 of mouse *Punc* cDNA, was labeled with [α^{32}P] dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was performed at a final stringency of 1.0 X SSCP, 0.1% SDS, 65°C. A fragment of 13.0 kb was detected in *Pst*I digested C57BL/6J DNA and a fragment of 5.1 kb was detected in *Pst*I digested *M. spretus* DNA. The presence or absence of the 5.1 kb *Pst*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Punc* including *Tnfsf5, Il1rak,* and *Ar* has been reported previously (Centanni*et al.*, 1998). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to account for the allele distribution patterns.

In Situ Hybridization

In situ hybridization was performed as described (Marquis, *et al.*, 1995) Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 1135 to 1509 of *Punc*.

Results

In order to isolate regulatory molecules potentially involved in mammary development and carcinogenesis, a degenerate RT-PCR based screen was performed to identify protein kinases expressed in murine breast cancer cell lines and in the mammary gland during development (Chodosh, *et al.*, 1999; Gardner, *et al.*, Submitted; Stairs, *et al.*, 1998). This screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, three of which were novel.

One of these novel kinases, originally called *Bstk3*, is the subject of this study. *Bstk3* was subsequently found to be upregulated in the mammary gland during pregnancy and to be expressed in a punctate pattern in the mammary epithelium. Therefore, *Bstk3* was renamed *Punc*, for Pregnancy-upregulated, nonubiquitous CaM kinase, to reflect its unique temporal and spatial expression pattern in the mammary gland.

Isolation of cDNA Clones Encoding *Punc*

Of approximately 1500 clones examined in the context of a screen for expressed protein kinases, a single clone corresponding to *Bstk3* was isolated from the mammary glands of mice undergoing early postlactational regression. In order to isolate the full-length mRNA transcript from which *Bstk3* was derived, this initial *204* bp RT-PCR product was used to screen a murine brain cDNA library. Three cDNA clones ranging from 1455 to 1554 nucleotides in length were isolated by these means. All three clones were completely sequenced and were found to differ only in their respective 5' untranslated regions (UTR) (Fig. 1). The sequence of each cDNA clone contains the entire 204 bp RT-PCR fragment, *Bstk3*, as well as a 1029 nucleotide open reading frame (ORF) and a 420 bp 3'-UTR possessing a polyadenylation signal and poly(A) tract (Fig. 1A).

Inspection of the nucleotide sequence surrounding the putative initiation codon at nucleotide 105 of the longest clone, U7, reveals matches with the Kozak translational initiation consensus

sequence at positions -1, -3, -5, and -6 (Kozak, 1987; Kozak, 1991). Conceptual translation of the *Punc* ORF yields a 343 amino acid polypeptide of predicted molecular weight 38.6 kDa. The coding sequence for *Punc* can be divided into a 14 amino acid unique amino-terminal segment, a 256 amino acid kinase catalytic domain, a 41 amino acid regulatory domain and a 32 amino acid unique carboxyl-terminal region. The *Punc* kinase catalytic domain contains all of the amino acid motifs conserved among serine/threonine kinases.

In order to determine whether the length of the cDNA clones encoding *Punc* are consistent with the size of the *Punc* mRNA transcript, Northern hybridization was performed. Due to potential cross-hybridization between *Punc* and homologous CaM kinase family members, Southern hybridization was used to confirm the specificity of a probe generated from the 3'-UTR of *Punc* (data not shown). This *Punc*-specific probe was used for Northern hybridization analysis performed on poly(A)$^+$ RNA isolated from adult murine brain. Consistent with the lengths of the isolated *Punc* cDNA clones, this analysis revealed an mRNA transcript approximately 1.6 kb in length.

In order to confirm the coding potential of the *Punc* ORF, *in vitro* transcription/translation was performed in the presence of ^{35}S-methionine using each of the three *Punc* cDNA clones as template. In each case, incubation of plasmid DNA with reticulocyte lysate yielded a single labeled polypeptide species of approximately 38 kDa, consistent with the predicted *Punc* ORF (Fig. 2B). This demonstrates that the predicted initiation codon is capable of functioning as a translation initiation site. Since clone U7 contains multiple in-frame termination codons upstream of this putative initiation codon, these findings suggest that we have isolated the entire *Punc* coding sequence. However, since the alternate 5'-UTR sequence present in clone V1 does not contain an upstream termination codon, we cannot exclude the possibility that alternate polypeptides encoded by the *Punc* locus exist that have distinct amino terminal sequences.

Homology to Related Protein Kinases

Multiple sequence alignment of Punc kinase catalytic subdomains I-XI was used to determine the homology between Punc and other CaM kinases (Fig. 3)(Hanks and Quinn, 1991; Hanks, *et al.*, 1988). Punc lies within the group of multifunctional CaM kinases and shares highest homology with CaMKI. Within the kinase domain, Punc is 70% identical to CaMKI, 50% identical to CaMKIV, and approximately 45% identical to members of the CaMKII subfamily. Punc is also homologous to members of the CaM kinase family in the regulatory domain, although the extent of homology is lower than that found in the catalytic domain. While Punc is most closely related to CaMKI in both the catalytic and regulatory domains, the homology between Punc and CaMKI is significantly lower than that between CaMKII subfamily members, most of which exhibit greater that 90% amino acid identity in the catalytic and regulatory domains. Moreover, outside of these conserved functional domains, the amino- and carboxyl-terminal regions of Punc bear no significant homology to CaMKI, CaMKIV, or other CaM kinase family members.

Chromosomal Localization

The chromosomal location of murine *Punc* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J x *Mus spretus)*F_1 X C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a cDNA probe specific for the 3'-UTR of *Punc*. The 5.1 kb *Pst*I *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Punc* locus in backcross mice. The mapping results indicated that *Punc* is located in the central region of the mouse X chromosome linked to *Tnfsf5, Il1rak*, and *Ar*. Although 106 mice were analyzed for every marker and are shown in the segregation analysis (Figure 4), up to 142 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using

the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere- *Tnfsf5* – 15/137 – *Punc* – 0/134 – *Il1rak* – 9/142 - *Ar.* The recombination frequencies expressed as genetic distances in centiMorgans (cM) ± the standard error are – *Tnfsf5* – 11.0 +/- 2.7 – [*Punc, Il1rak*] – 6.3 +/- 2.0 – *Ar.* No recombinants were detected between *Punc* and *Il1rak* in 134 animals typed in common suggesting that the two loci are within 2.2 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of the X chromosome with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *Punc* maps to a region of the composite map that lacks uncloned mouse mutations (data not shown).

The central region of the mouse X chromosome shares a region of homology with the long arm of the human X chromosome (summarized in Figure 4). In particular, *Il1rak* has been mapped to Xq28. The close linkage between *Il1rak* and *Punc* in mouse, suggests that the human homologue of *Punc* will map to Xq28, as well.

Analysis of *Punc* mRNA expression

In order to begin to determine the biological role of *Punc*, the developmental expression pattern of *Punc* mRNA was analyzed during murine embryogenesis. Northern hybridization analysis was performed on poly(A+) RNA isolated from embryos during early, mid, and late gestation using a *Punc*-specific probe (Fig. 5A). Compared to mRNA expression levels in early embryogenesis, steady-state *Punc* mRNA levels are markedly upregulated in the embryo during midgestation and remain elevated through embryonic day 18.5.

In order to investigate the spatial expression pattern of *Punc* expression during fetal development, *in situ* hybridization analysis was performed on embryonic sections at day 14.5 of gestation using an ^{35}S-labeled *Punc*-specific antisense probe. The spatial pattern of *Punc*

expression in the embryo at midgestation reveals tissue-specific expression of *Punc* with highest levels of expression detected in developing bone, the developing central nervous system, including periventricular regions and the trigeminal ganglion, and the outer lining of the stomach.

The expression profile of *Punc* in organs of the adult mouse was determined by RNase protection analysis. As in the embryo, *Punc* expression in the adult mouse is highest in brain. In addition, moderate to low levels of *Punc* expression are detected in hormonally responsive tissues such as uterus, ovary, testis, and mammary gland, as well as in other tissues such as stomach, heart and skeletal muscle. Lower, but detectable levels of *Punc* expression were observed in thymus, spleen, duodenum, and lung.

Finally, the spatial expression pattern of *Punc* in adult murine tissues was determined by *in situ* hybridization analysis. Interestingly, within expressing tissues, *Punc* mRNA was detected within only a subset of cells. In the brain, *Punc* expression is highest in the dentate gyrus and CA1-3 regions of the hippocampus (Fig. 6B, C, F, &G). *Punc* is also expressed at relatively high levels in the cortex and is markedly heterogeneous with highly expressing cells found adjacent to non-expressing cells (Fig. 6 J, K). *Punc* is expressed throughout the ovary, but is preferentially localized in the thecal cell layers immediately surrounding the corpora lutea (Fig. 6 H, I). In the testis, *Punc* is expressed at high levels in mature spermatids residing at the center of seminiferous tubules and, to a lesser extent, in cells located adjacent to the basement membrane (Fig. 6 D, E). In the dorsolateral prostate, *Punc* mRNA is detected in a stromal layer of cells immediately surrounding the prostatic epithelial ducts. As in other tissues, *Punc* expression in this compartment is spatially heterogeneous (Fig. 6 L, M).

Discussion

We have described the cloning, chromosomal localization and developmental expression pattern of *Punc*, a new member of the CaM kinase family of serine/threonine kinases that is most closely related to CaMKI. *Punc* expression during embryogenesis is developmentally regulated and tissue-specific with highest levels of expression detected in the developing brain, bone and gut. Interestingly, *Punc* expression in adult animals is both tissue-specific and markedly heterogeneous. *Punc* expression is restricted to specific compartments within several tissues, in which *Punc* expressing cells are found adjacent to non-expressing cells. The *Punc* locus maps to the mouse X chromosome in a region syntenic with human chromosome Xq28, a region associated with several mental retardation syndromes. In aggregate, our data suggest that *Punc* may be involved in a variety of developmental processes.

Within the catalytic and regulatory domains conserved among all CaM kinases, *Punc* is most closely related to the multifunctional CaM kinases, CaMKI, CaMKIV, and members of the CaMKII subfamily. CaMKI is a monomeric kinase that is expressed in multiple tissues and is reported to phosphorylate several substrates including synapsin, the cystic fibrosis transmembrane conductance regulator, and transcription factors such as the cyclic AMP response element binding protein, CREB and ATF-1 (Lukas, *et al.*, 1998; Nairn and Picciotto, 1994; Nastluk and Nairn, 1996; Sheng, *et al.*, 1991). CaMKIV is located in the nucleus and has been proposed to mediate CaM-induced changes in gene expression (Jensen*et al.*, 1991a; Sun*et al.*, 1996). In contrast to CaMKI and IV which function as monomers, CaMKII forms 300-600 kD multimers comprised of different combinations of α, β, γ, and δ subunits (Schulman, 1993). While the α and β subunits are expressed predominantly in brain, the γ and δ CaMKII subunits are expressed ubiquitously (Hanley, *et al.*, 1987; Lin, *et al.*, 1987; Tobimatsu and Fujisawa, 1989; Tobimatsu, *et al.*, 1988).

Functional analysis of CaM kinase mutants as well as crystal structure information have been used to define amino acids involved in the regulation of this family of molecules (Goldberg*et al.*, 1996; Haribabu, *et al.*, 1995; Yokokura, *et al.*, 1995). Carboxyl-terminal to their catalytic domain, CaM kinases posses a regulatory region that is comprised of an autoinhibitory domain and

a CaM binding domain. Current evidence suggests that CaM binding disrupts an interaction between the autoinhibitory domain and the kinase catalytic domain, thereby resulting in kinase activation. In contrast to other CaM kinases, the activity of CaMKI and CaMKIV is dependent upon phosphorylation by a CaM-dependent kinase kinase, CaMKK (Haribabu, *et al.*, 1995; Tokumitsu*et al.*, 1994; Tokumitsu*et al.*, 1995). Since Punc may be regulated in a manner similar to other CaM kinases, particularly CaMKI, structural homologies between Punc and other CaM kinases should help elucidate the mechanisms by which Punc activity is regulated.

The homology between *Punc* and CaMKI raises the issue of whether *Punc* should be classified as a CaMKI family member. Currently, the only widely recognized CaM kinase subfamily is that of CaMKII. Primary amino acid sequences of CaMKII subfamily members are greater than 90% identical in the catalytic and regulatory domains and actually function together in a multiprotein complex. In contrast, while the homology between *Punc* and CaMKI is greater than that between *Punc* and other CaM kinases, at 70% the homology between *Punc* and CaMKI is significantly less than the 90% identity observed between most CaMKII family members. Moreover, there is currently no evidence to suggest that CaMKI family members function as subunits in a manner analogous to CaMKII subfamily members. As such, while *Punc* is most homologous to CaMKI, it is unclear at present whether this kinase should be classified as a CaMKI family member.

While this work was in progress, the rat homologue of *Punc* was described and shown to be expressed as two isoforms, tentatively named CaMKIβ1 and CaMKIβ2 (Naito, *et al.*, 1997). Similar to the clones isolated for *Punc*, CaMKIβ1 and CaMKIβ2 differ in their 5' UTR regions and are homologous to *Punc* clones V1 and U7, respectively. However, unlike the full-length clones isolated for *Punc*, CaMKIβ1 contains a unique carboxyl-terminal coding region that appears to result from an alternative splicing event. Whether this form exists in the mouse remains to be determined. By Northern hybridization analysis using a probe encompassing portions of the highly conserved kinase domain and regulatory region of CaMKIβ isoforms, a 1.8 kb band was detected exclusively in brain and a 4.0 kb band was detected in all other tissues. RT-PCR analysis

detected approximately equal levels of CaMKIβ1 in all tissues examined in the rat, including tissues that express low to undetectable levels of *Punc* in the mouse as determined by RNase protection analysis using a probe specific for the 3'-UTR of *Punc*. Insofar as the tissue-specific expression pattern of *Punc* has been confirmed by *in situ* hybridization analysis, it is possible that the cross-hybridization of the CaMKIβ probe used for Northern hybridization with other CaM kinases or the nonlinear nature of RT-PCR may underlie the discrepancy between expression of *Punc* and that reported for its rat homologue, CaMKIβ.

Several CaM kinases are expressed at high levels in the brain including CaMKI, CaMKII, and CaMKIV (Lukas, *et al.*, 1998; Miyano*et al.*, 1992; Picciotto, *et al.*, 1995). In fact, it has been estimated that CaMKII accounts for approximately 2% of all protein found in the hippocampus (Hanson and Schulman, 1992). It is therefore perhaps not surprising that *Punc* is also expressed at high levels in the murine brain both in the adult and during embryogenesis. In the adult brain, *Punc* is expressed at highest levels in the hippocampus and dentate gyrus, two areas of the brain involved in learning and memory.

While expression of CaMKI, CaMKIV and isoforms of CaMKII has been reported in tissues other than the brain, a physiological role for these enzymes in other tissues has not been described. Similar to these multifunctional kinases, *Punc* is expressed in a variety of tissues other than the brain and in most tissues examined, is expressed in a spatially heterogeneous manner with expression restricted to a subset of cells. Although little is known about the role of CaM or CaM kinases in development, evidence suggests that both CaM and CaM kinases may play such a role. Point mutations in the Drosophila calmodulin gene result in defects in development with phenotypes ranging from pupal lethality to ectopic wing vein formation and melanotic scales on the cuticle (Nelson*et al.*, 1997). Additionally, CaMKIV has been implicated in T cell development based upon its regulation in the thymus during T cell development (Krebs*et al.*, 1997). The observation that *Punc* expression is developmentally regulated and spatially restricted to distinct compartments of the ovary, testis, prostate and brain suggests that *Punc* may play a biological role

in these tissues. As such, the elucidation of signaling pathways in which *Punc* is involved may shed light on the broader physiological role played by CaM kinases.

We have mapped the murine gene encoding *Punc* to within 2.2 cM of *Il1rak* in the central region of the X chromosome. The observation that *Il1rak*, as well as markers that lie within 2.2 cM on either side of *Il1rak,* have been mapped to human chromosome Xq28, strongly suggests that the human homologue of *Punc* will map to Xq28 as well. Chromosome Xq28 is one of the most densely mapped regions of the human chromosome and several distinct mental retardation syndromes including Fragile X and X-linked mental retardation (XLMR) have been mapped to this region (Knight*et al.*, 1993; Lubs, *et al.*, 1999). Interestingly, the only biological role described for any of the multifunctional CaM kinases is that of CaMKII in learning and memory, suggesting that CaM kinases may play an important role in signal transduction pathways controlling cognitive function (Silva, *et al.*, 1992a; Silva, *et al.*, 1992b; Soderling, 1993). In addition to mental retardation, many of these syndromes include phenotypes such as short stature, cleft palate, altered hand or digit size, and sterility (Lubs, *et al.*, 1999). Given that *Punc* is expressed at high levels in the brain, developing bone, ovary and testis, it will be interesting to determine whether *Punc* plays a role in one or more of these Xq28-linked syndromes.

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Figure Legends

Figure 1. Nucleotide and deduced amino acid sequence of *Punc*. A. Composite nucleic acid sequence and conceptual translation of full-length *Punc* cDNA. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in bold on the right. A shaded box indicates the kinase catalytic domain and a hatched box denotes the putative regulatory region. The in-frame upstream termination codons in the 5'-UTR and the putative polyadenylation sequence in the 3'-UTR are underlined by thin and thick lines, respectively. The putative initiation codon is boxed and an asterisk denotes the stop codon. The regions corresponding to the degenerate oligonucleotides used to initially clone *Bstk3* are indicated by arrows. B. 5'-UTR sequences of three full-length cDNA clones encoding *Punc*. Nucleotide coordinates relative to each clone are shown to the right. Upstream in-frame termination codons are underlined and the putative initiation codons are boxed.

Figure 2. Expression, identification and coding potential of *Punc*. A. Northern hybridization analysis of 4 μg poly(A)$^+$ RNA isolated from adult murine brain hybridized with a 3'-UTR probe specific for *Punc*. The relative migration of RNA size markers is indicated. B. *In vitro* transcription/translation reactions performed using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and 1 μg of template consisting of a full-length *Punc* cDNA clone (V1, U7, or Q3) or, a cDNA plasmid encoding an unrelated kinase (−) as a negative control. IVT reactions were resolved on a 10% SDS-PGE gel and subject to autoradiography. The relative migration of molecular weight markers is indicated.

Figure 3. Homology between *Punc* and CaM kinase family members. Phylogenetic tree illustrating the relationship of *Punc* kinase catalytic subdomains I-XI to other CaM kinase family members. Analysis and depiction of results was performed using the ClustalX multisequence alignment program and DendroMaker 4.0. Evolutionary relationships are

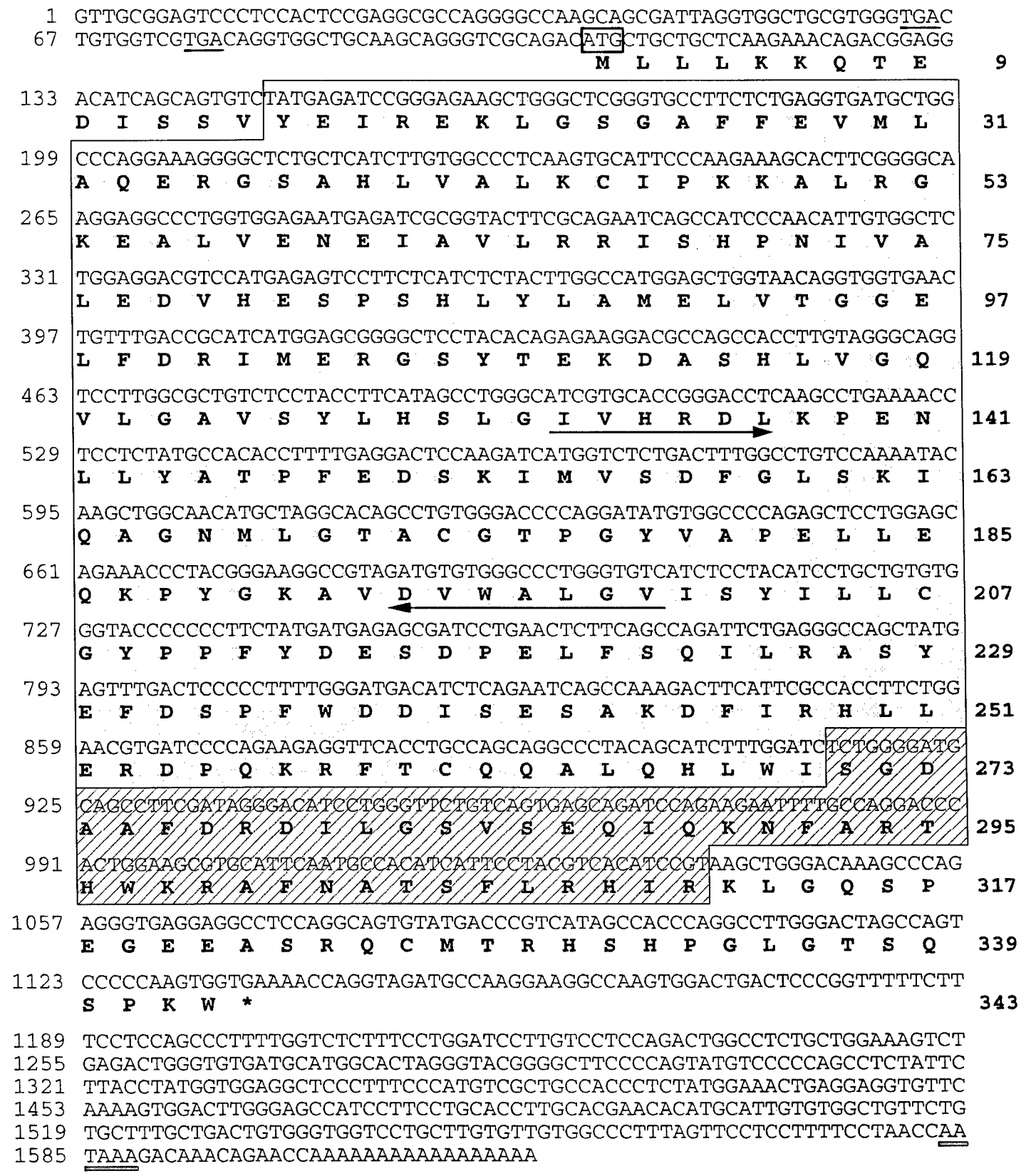
proportional to horizontal branch distances. Database accession numbers used are: A30355 (CaMKIIα); A34366 (CaMKIIδ); A26464 (CaMKIIβ); A31908 (CaMKIIγ); M64757 (CaMKIV); L26288 (CaMKI); X07320 (PhK-γ); A41674 (SmMLCK); X76104 (DAPK).

Figure 4. *Punc* maps in the central region of the mouse X chromosome. *Punc* was placed on the mouse X chromosome by interspecific backcross analysis. The segregation patterns of *Punc* and flanking genes in 106 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 106 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J x *M. spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial X chromosome linkage map showing the location of *Punc* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

Figure 5. Expression of *Punc* during murine embryogenesis. A. Northern hybridization analysis of 3 μg of poly(A)$^+$ RNA isolated from day E6.5, E13.5, and E18.5 embryos hybridized with a ^{32}P-labeled DNA probe specific for the 3'-UTR of *Punc*. B. *In situ* hybridization analysis of *Punc* mRNA expression in the murine embryo. Sections of embryos at day 14.5 of gestation were hybridized with an ^{35}S-labeled *Punc* antisense RNA probe. No signal over background was detected in serial sections hybridized with a sense *Punc* probe. bo, bone; bt, basal telencephalon; fv, fourth ventricle; li, liver; lv, lateral ventricle; st, stomach; tg, trigeminal ganglion; wf, whisker hair follicle. Magnification 10X. Exposure time 6 weeks.

Figure 6. Expression of *Punc* in adult tissues. A. RNase protection analysis of *Punc* mRNA expression in tissues of the adult mouse. RNase protection analysis was performed using 30 μg of RNA isolated from the indicated murine tissues using antisense RNA probes specific for *Punc* as well as for β-actin as an internal control. tRNA was used as a negative control for non-specific hybridization. B-M, Spatial localization of *Hunk* expression in tissues of the adult mouse. Bright-field (B, D, F, H, J, L) and dark-field (C, E, G, I, K, M) photomicrographs of *in situ* hybridization analysis performed on sections of brain (B, C, F, G, J, K), testis (D, E), ovary (H, I), and prostate (L, M), hybridized with an ^{35}S-labeled *Punc* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Punc* probe. Arrows and arrowheads indicate *Punc* expressing and *Punc* non-expressing cells, respectively. bm, basement membrane; CA1 and CA3, regions of the hippocampus; co, cortex; d, duct; dg, dentate gyrus; fo, follicle; se, semeniferous tubule; sp, spermatids; st, stroma. Magnification: 10X (B,C), 300X (D-M). Exposure times were 6-7 weeks.

A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M L L L K K Q T E 9
133 ACATCAGCAGTGTCTATGAGATCCGGGAGAAGCTGGGCTCGGGTGCCTTCTCTGAGGTGATGCTGG
D I S S V Y E I R E K L G S G A F F E V M L 31
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A Q E R G S A H L V A L K C I P K K A L R G 53
265 AGGAGGCCCTGGTGGAGAATGAGATCGCGGTACTTCGCAGAATCAGCCATCCCAACATTGTGGCTC
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V L G A V S Y L H S L G I V H R D L K P E N 141
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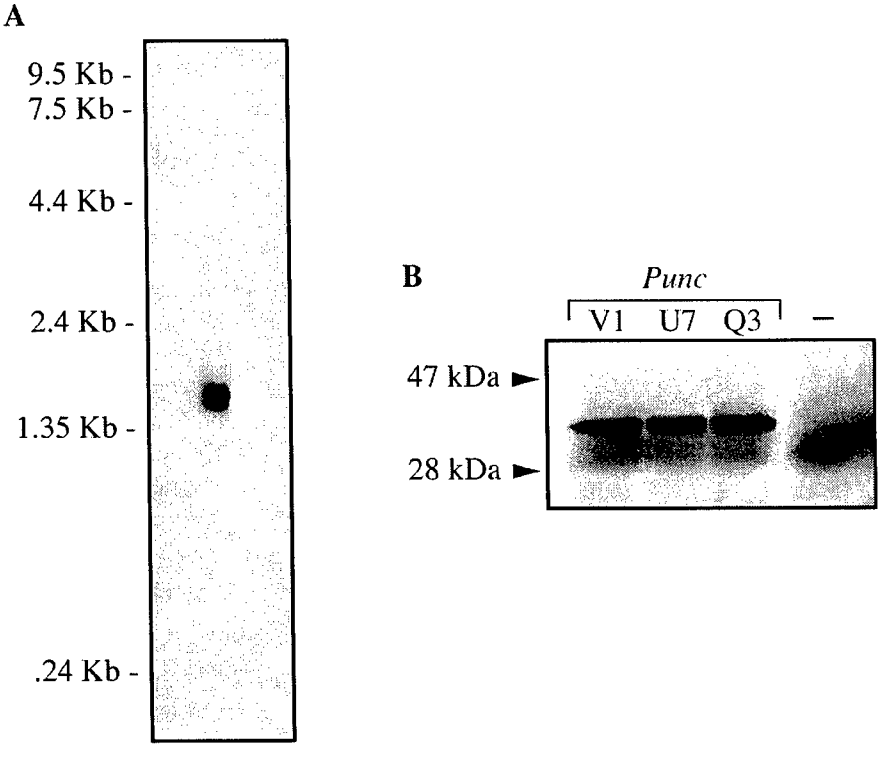
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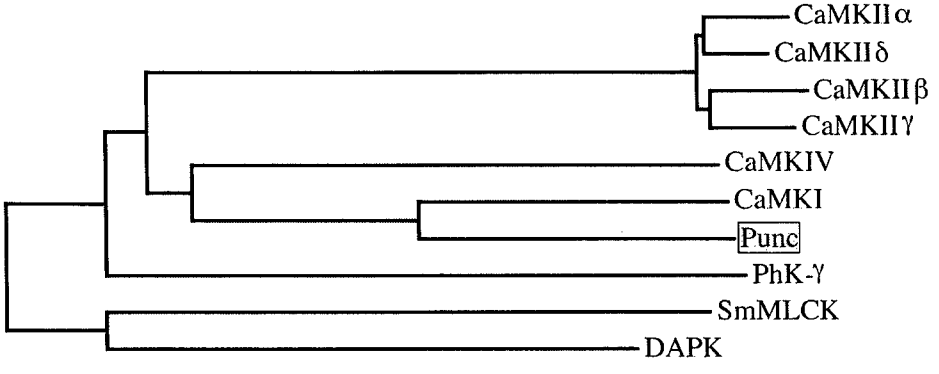
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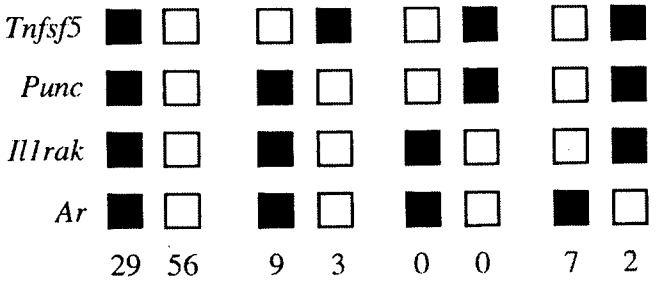
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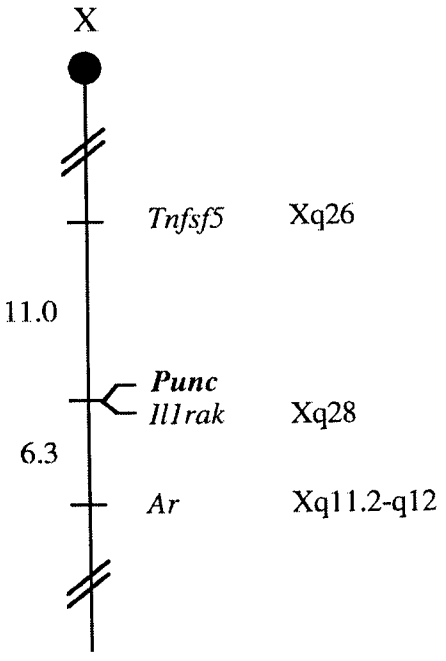
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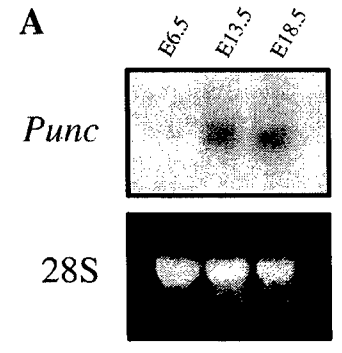
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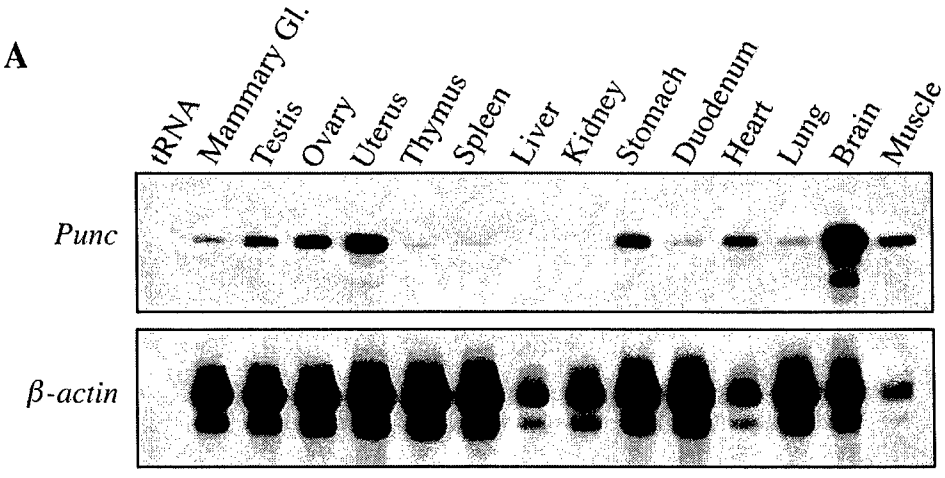
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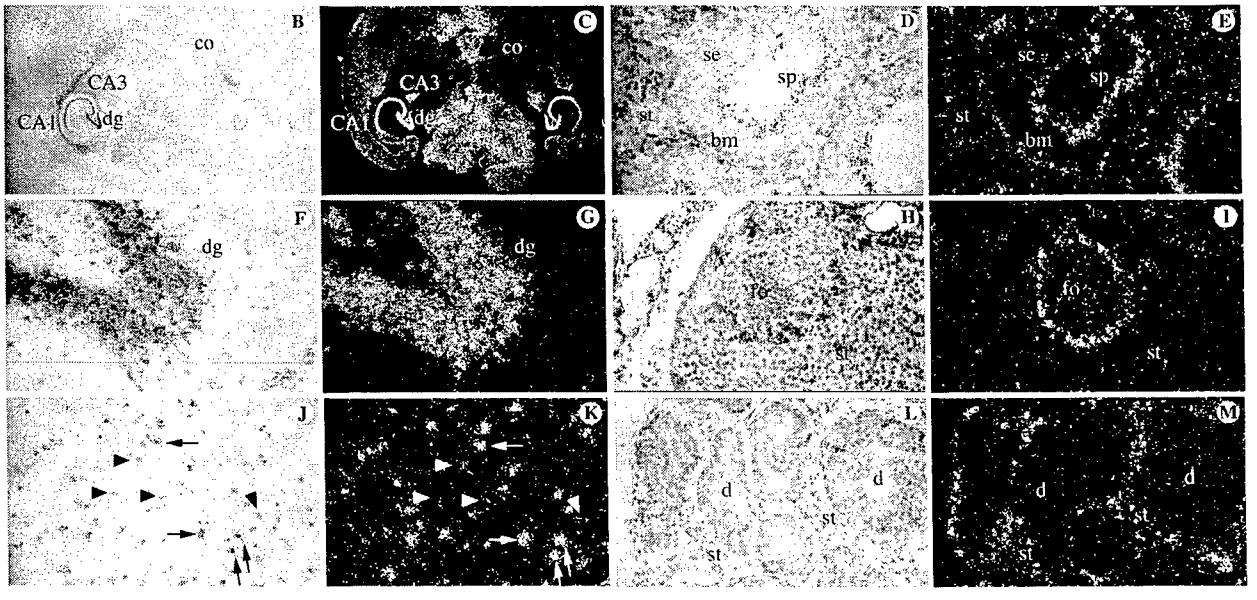
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Developmental Role of Hunk in Mediating Pregnancy-Induced Changes in the Mammary Gland

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Running title: Role of Hunk in Mammary Development

Key words: mammary gland, protein kinase, transgenic, cell differentiation, hormone

SUMMARY

Numerous observations in humans and in rodent model systems indicate that estradiol and progesterone regulate key phases of mammary gland development and play a central role in the pathogenesis of breast cancer. Therefore, molecules whose expression is influenced by hormones and which play a role in development may also contribute to mammary carcinogenesis. In a screen aimed at identifying protein kinases involved in mammary development and carcinogenesis, we previously identified a novel SNF1-related serine/threonine kinase, *Hunk*. In this report we provide evidence implicating *Hunk* in murine mammary development and carcinogenesis, as well as in the response of the mammary epithelium to estradiol and progesterone during pregnancy. Interestingly, in mammary epithelial cell lines derived from different transgenic mouse models of breast cancer, *Hunk* is expressed in an oncogene-restricted manner, with high levels of expression in cell lines derived from mammary tumors initiated by *neu* and v-Ha-*ras,* and undetectable levels in cell lines initiated by c-*myc*. *Hunk* expression in the mammary gland is developmentally regulated with highest levels of expression occurring during early pregnancy, and is spatially restricted within a subset of mammary epithelial cells throughout postnatal development. Furthermore, treatment of mice with 17ß-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in the mammary epithelium, suggesting that this kinase may be regulated by ovarian hormones. Consistent with tightly regulated pattern of *Hunk* expression during development, mammary glands from transgenic mice engineered to misexpress *Hunk* in the epithelium during late pregnancy and lactation fail to undergo normal lobuloalveolar development. These observations suggest that *Hunk* may be involved in mediating pregnancy-induced changes in the mammary gland that occur in response to ovarian hormones, is expressed in a biologically important subset of mammary epithelial cells, and may be involved in the process of mammary carcinogenesis.

INTRODUCTION

A wealth of epidemiologic evidence indicates that ovarian hormones play a critical role in the etiology of breast cancer (Kelsey et al., 1993). Specifically, the observations that early menarche, late menopause and postmenopausal hormone replacement therapy are each associated with an increased breast cancer risk, whereas early oophorectomy is associated with a decreased breast cancer risk, have led to the hypothesis that a woman's risk of developing breast cancer is proportional to her cumulative exposure to estradiol and progesterone (Henderson et al., 1988; Pike et al., 1993). As such, elucidating the mechanisms by which hormones contribute to breast carcinogenesis is a central goal of breast cancer research.

In addition to their roles in the pathogenesis of breast cancer, estradiol and progesterone are the principal steroid hormones responsible for regulating the development of the mammary gland during puberty, pregnancy and lactation (Topper et al., 1980). For example, estradiol action is required for epithelial proliferation and ductal morphogenesis during puberty, whereas progesterone action is required for ductal arborization and alveolar differentiation during pregnancy (Topper et al., 1980; Bocchinfuso et al., 1997; Humphreys et al., 1997). The effects of estradiol and progesterone in a given tissue are ultimately determined by the activation and repression of their respective target genes. As such, understanding the effects of estradiol and progesterone in the breast will require the identification of the downstream targets of these hormones and elucidation of such downstream molecules will likely identify proteins involved in mammary development and mammary carcinogenesis.

Protein kinases function as molecular switches in transduction pathways critical to cellular processes such as proliferation and differentiation. Accordingly, aberrant expression or mutations in several members of the protein kinase family have been shown to be involved in the pathogenesis of breast cancer both in humans and in rodent model systems (Cardiff et al., 1993). Moreover, overexpression of protein kinases such as *ErbB2/neu* in human breast cancers has been shown to provide prognostic information relevant to clinical outcome and response to therapy (Slamon et al., 1987; Slamon et al., 1989; Klijn et al., 1993). Further studies of the function of

protein kinases in the breast may reveal significant insights into how the decision to proliferate or differentiate is made in mammary epithelial cells.

Given the importance of protein kinases in development and carcinogenesis in a broad range of tissues, we previously carried out a PCR-based screen to identify kinase family members expressed during mammary gland development and in mammary epithelial cell lines derived from four different transgenic mouse models of breast cancer (Chodosh et al., 1999). This screen identified 41 kinases including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel.

One of these novel kinases, Hunk, is an 80 kDa putative serine/threonine kinase that bears homology to the SNF1 protein kinase family (Gardner et al., submitted). Members of this family, including SNF1 from *S. cerevisiae* and AMP-Activated Protein Kinase in mammals, regulate a variety of cellular processes including metabolic changes that occur in response to nutritional and environmental stresses (Hardie et al., 1994). Other SNF1-related kinases, including PAR-1, C-TAK1/p78, MARK1, MARK2, and Msk, have been implicated in the regulation of proliferation and development (Ruiz et al., 1994; Guo et al., 1995; Bohm et al., 1997; Peng et al., 1997). For instance, C-TAK1/p78 is able to phosphorylate and inactivate Cdc25c, thereby preventing activation of Cdc2 and presumably inhibiting entry of cells into mitosis (Peng et al., 1997; Peng et al., 1998). Consistent with this hypothesis, expression of C-TAK1/p78 protein is downregulated in adenocarcinomas of the pancreas (Parsa, 1988). Mutations in the *C. elegans* SNF1 family member, *PAR-1,* disrupt P-granule localization and result in the organism's inability to establish polarity during embryogenesis (Guo et al., 1995). Analogously, a mammalian homologue of PAR-1, MARK2/Emk, is asymmetrically localized in polarized epithelial cells and expression of a dominant negative form of MARK2 disrupts cell polarity (Bohm et al., 1997). These data suggest a role for SNF1 family members in both development and carcinogenesis in higher eukaryotes.

We have previously described the cloning, activity, and chromosomal localization of *Hunk* and have characterized its expression pattern both in the murine embryo and in adult organs (Gardner et al., submitted). The gene encoding Hunk is located on the distal arm of mouse

chromosome 16 in a region syntenic with human chromosome 21q22 within the Down Syndrome minimal critical region. We have shown that *Hunk* mRNA expression is temporally and spatially regulated during embryonic development. During mid-gestation, *Hunk* is expressed at high levels within a subset of developing organs in the embryo, and is down-regulated in some, but not all, tissues prior to parturition. As in the embryo, *Hunk* expression in the adult mouse is tissue-specific. Interestingly, *Hunk* mRNA expression is restricted to specific compartments within expressing organs and it is further restricted to subsets of cells within those compartments. For example, *Hunk* expression in the duodenum is limited to a subset of epithelial cells in the duodenal crypts, and little or no expression is observed in the more differentiated cells of the duodenal epithelium. In aggregate, these observations suggest that *Hunk* expression is restricted to particular cellular subtypes within a variety of tissues, and support a role for Hunk in developmental processes in multiple tissues.

In this report, we investigate the role of *Hunk* in mammary gland development and carcinogenesis. *Hunk* mRNA expression in the mammary gland is spatially restricted to subsets of epithelial cells during all stages of postnatal development. Surprisingly, *Hunk* is expressed in an oncogene-restricted manner in mammary epithelial cell lines derived from different mouse models of breast cancer. Our data further demonstrate that *Hunk* expression is developmentally regulated in the mammary gland with highest levels of expression detected early in pregnancy. Moreover, treatment of mice with estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in the mammary epithelium, suggesting that *Hunk* upregulation during early pregnancy is due to ovarian hormones. Finally, misexpression of *Hunk* in the mammary glands of MMTV-*Hunk* transgenic mice results in the failure of the mammary epithelium to terminally differentiate during late pregnancy and lactation.

Taken together, our data suggest that *Hunk* contributes to mammary development by regulating pregnancy-induced changes that occur in response to estrogen and progesterone. Our findings further suggest that *Hunk* may be expressed in biologically important subsets of epithelial cells in the breast, and may be involved in the process of mammary carcinogenesis.

MATERIAL AND METHODS

Generation of MMTV-Hunk Transgenic Mice

A full-length cDNA clone, G3, encoding *Hunk* was digested with SmaI and SpeI to liberate a 3.2 kb fragment containing the entire coding sequence for Hunk (accession # AF167987). This fragment was cloned downstream of the MMTV LTR into pBS-MMTV-pA vector which consists of a pBS backbone, the MMTV LTR upstream of the H-ras leader sequence, followed by a multiple cloning site directly upstream of the SV40 splice site and polyadenylation signal (E. Gunther, unpublished). Linearized plasmid DNA was injected into fertilized oocytes harvested from superovulated FVB mice. Mice were genotyped by Southern blotting and by PCR using two sets of primers comprised of one primer pair located in the SV40 portion of the transgene, and a second primer pair spanning the junction beween the *Hunk* cDNA and SV40 sequences. Primers designed to amplify the *Gapdh* locus were used as a positive control for PCR reactions. Oligonucleotide primer sequences were mGAPDH.466F: CTCACTCAAGATTGTCAGCAATGC; mGAPDH.1056B: AGGGTTTCTTACTCCTTGGAGGC; SV40pAL: CCTTAAACGCCTGGTGCTACGC; SV40pAR: GCAGTAGCCTCATCATCACTAGATGG; A32-G3.2857F: CTTTCTTTTTCCCCTGACC; POLYA.XBAB: ACGGTGAGTAGCGTCACG.

Five potential founder mice were identified harboring the MMTV-*Hunk* transgene in tail-derived DNA. Tail-derived DNA was prepared as described (Hogan et al., 1994). All founders were found to pass the transgene to offspring in a Mendelian fashion with the exception of one founder that passed the transgene exclusively to males. The remaining four founder lines were screened for transgene expression by both Northern hybridization and RNase protection analysis.

All animals that were transgene-positive by PCR genotyping, but that were found not to express the MMTV-*Hunk* transgene, were subsequently analyzed by Southern hybridization to confirm transgene presence and the expected MHK3 integration site. Tail-derived genomic DNA was digested with SpeI, electrophoresed on a 0.8 % agarose gel, and transfered onto Genescreen

Plus membrane (Dupont/NEN). Hybridization was performed according to standard methods using a probe specific to the SV40 portion of the transgene.

Animal and Tissue Preparation

FVB mice were housed under barrier conditions with at 12-hour light/dark cycle. Mammary glands from pregnant females were harvested at specified time points following timed matings. Day 0.5 was defined as noon of the day on which a vaginal plug was observed. For chronic hormone treatment experiments, adult female FVB mice were subject to bilateral oophorectomy and allowed to recover for two weeks prior to hormonal injections administered as previously described (Marquis et al., 1995). For 24-hour hormone administration experiments, four month-old virgin female FVB mice were injected subcutaneously with either phosphate buffered saline (PBS) as a control or a combination of 5 mg progesterone in 5% gum arabic and 20 μg of 17β-estradiol in PBS. Four animals from each treatment group were sacrificed 24±1 hours following injection. Tissues used for RNA analysis were snap frozen on dry ice and tissues used for in situ hybridization analysis were embedded in OCT compound.

For whole mount analysis, number four mammary glands were spread on glass slides and fixed for 24 hours in 10% neutral buffered formalin. Glands were then placed in 70% ethanol for 15 minutes followed by 15 minutes in deionized water before staining in 0.05% Carmine/0.12% Aluminum Potassium Sulfate for 24-48 hours. Glands were dehydrated sequentially in 70%, 90% and 100% ethanol for 10 minutes each and then cleared in toluene or methyl salicylate overnight.

For histological analysis, mammary glands were fixed as above and transferred to 70% ethanol prior to paraffin embedding. 5 μm sections were cut and stained with hematoxylin and eosin.

RNA Preparation and Analysis

RNA preparation, Northern hybridization and labeling of cDNA probes was performed as previously described (Marquis et al., 1995). Probes for *Hunk* encompassed nucleotides 1149 to 3849 or nucleotides 275 to 793 of the full-length cDNA (accession # AF167987). Probes to milk

protein genes were β-Casein: nt 181-719 (accession #X04490); κ-Casein: nt 125-661 (accession #M10114); Lactoferrin: nt 993-2065 (accession #D88510); α-Lactalbumin: nt 174-700 (accession #M80909); WDNM1: nt 86-328 (accession #X93037); WAP: nt 131-483 (accession #X01158); and ε-Casein: nt 83-637 (accession #V00740).

Ribonuclease protection analysis was performed as described using body-labeled antisense riboprobes specific to nucleotides 276-500 of *Hunk* (accession #AF167987) and 1142-1241 of *ß-actin* (accession # X03672)(Marquis et al., 1995). In order to distinguish transgenic from endogenous *Hunk* expression in MHK3 animals, a probe template was generated spanning the 3' end of the *Hunk* cDNA and the 5' end of the SV40 polyadenylation signal sequence. A *ß-actin* antisense riboprobe was added to each reaction as an internal control. Signal intensities were quantitated using a phosphorimager (Molecular Dynamics).

In Situ Hybridization

In situ hybridization was performed as described (Marquis et al., 1995; Rajan et al., 1997) using a PCR template containing nucleotides 276 to 793 of *Hunk*. Exposure times were 6 weeks in all cases.

Tissue Culture

Cells were cultured in DMEM medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transformed cell lines were derived from mammary tumors or hyperplastic lesions that arose in MMTV-*c-myc*, MMTV-*int-2*, MMTV-*neu*/NT or MMTV-*Ha-ras* transgenic mice as described(Morrison et al., 1994). NIH 3T3, NMuMG, and CL-S1 cells were obtained from ATCC. HC11 cells were the kind gift of Nancy Hynes and Jeff Rosen.

Protein Analysis

Generation of anti-Hunk antisera, immunoblotting and immunoprecipitation were performed as described (Gardner et al., submitted). Protein was extracted from mammary glands by dounce homogenization in EBC buffer as described (Gardner et al., submitted). From these extracts, 500 μg of protein in 167 μl of EBC was precleared with 17 μl of 1:1 protein A-Sepharose in PBS for overnight at 4°C. Precleared lysates were incubated overnight at 4°C in EBC plus 5% Tween 20 (Biorad) with or without affinity-purified antisera raised against the carboxyl-terminus of Hunk (0.4 μg/ml). Immune complexes were precipitated by incubating with 40 μl of 1:1 Protein A-Sepharose in PBS for 1 hr at 4°C. Complexes were washed once with EBC plus 5% Tween 20, twice with EBC, and twice with PBS. One fifth of the precipitated complexes were subject to an in vitro kinase reaction as previously described with 5 μM ATP and 0.5 μg/μl histone H1 (Gardner et al., submitted). The remaining precipitate was electrophoresed on 10% SDS-PAGE gel, transferred onto PVDF membranes, and immunoblotted with an antibody against the carboxyl-terminus of Hunk as described (Gardner et al., submitted).

RESULTS

Hunk, initially termed *Bstk1*, was identified as a 207 bp RT-PCR product isolated from an epithelial cell line derived from a mammary adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh et al., 1999; Gardner et al., submitted). As described in this report, *Bstk1* was subsequently renamed *Hunk* (Hormonally upregulated *neu* tumor-associated kinase) to reflect the observation that *Bstk1* expression in the mammary gland is upregulated early in pregnancy and in response to ovarian hormones, and that *Bstk1* is preferentially expressed in mammary epithelial cell lines overexpressing the *neu* oncogene.

Hunk Expression is Developmentally Regulated in the Mammary Gland

RNase protection analysis was used to determine the temporal pattern of *Hunk* expression during the postnatal development of the murine mammary gland (Fig. 1A). Mammary glands were harvested from male FVB mice, virgin mice at developmental time points prior to puberty (2 wk), during puberty (5 wk), and following puberty (10 wk and 15 wk), as well as from mice during early, mid and late pregnancy (day 7, 14 and 20), lactation (day 9), and postlactational regression (day 2, 7 and 28). RNase protection analysis performed on total RNA prepared from these glands revealed that steady-state levels of *Hunk* mRNA are low and remain relatively constant throughout virgin development. During early pregnancy (day 7), when alveolar buds begin to rapidly proliferate and differentiate, *Hunk* mRNA levels undergo a transient but dramatic increase, and then return to baseline by mid-pregnancy (Fig. 1A and B). The apparent decline in *ß-actin* expression seen by RNase protection analysis during late pregnancy, lactation and early postlactational regression results from a dilutional effect due to the onset of large-scale milk protein gene expression (Gavin et al., 1992; Buhler et al., 1993; Marquis et al., 1995). Normalization of *Hunk* expression to *ß-actin* to control for these dilutional changes confirms that *Hunk* expression returns to baseline levels by mid-pregnancy and decreases further during lactation and early postlactational regression (Fig. 1B).

In order to determine whether the observed pregnancy-induced changes in *Hunk* mRNA expression levels represent global changes in expression throughout the mammary gland, or changes in expressing subpopulations of cells, in situ hybridization was performed (Fig. 1C). Consistent with the results from RNase protection analysis, in situ hybridization confirmed that *Hunk* expression in the mammary gland is highest at day 7 of pregnancy and decreases progressively throughout the remainder of pregnancy and lactation. This analysis also revealed that *Hunk* is expressed exclusively in the epithelium throughout mammary gland development and that *Hunk* upregulation during pregnancy is due to upregulation in a subset of epithelial cells rather than global upregulation throughout the epithelium (Fig. 1C and data not shown).

Interestingly, as in other organs of the adult mouse, *Hunk* expression in the mammary gland is spatially restricted to subsets of epithelial cells in which highly expressing cells are located adjacent to non-expressing cells (Figs.1C and 2). This pattern is particularly striking in terminal end buds and epithelial ducts of the pubertal gland (Fig. 2). This punctate expression pattern differs from the homogeneous expression of most genes examined in the mammary gland (data not shown).

Hunk Expression is Regulated by Ovarian Hormones

The observation that *Hunk* mRNA levels in the mammary gland increase during pregnancy suggested that the expression of this gene may be modulated by estrogen and progesterone. In order to test this possibilty, oophorectomized FVB mice were treated for fourteen days with 17ß-estradiol alone, progesterone alone, or a combination of both hormones. Intact (sham) and oophorectomized, non-hormone treated (control) animals were used for comparison.

Hunk mRNA levels were quantitated by RNase protection analysis of RNA prepared from mammary glands or uteri pooled from at least 10 animals in each experimental group (Fig. 3). In the mammary gland, steady-state *Hunk* mRNA levels decreased approximately four-fold relative to intact mice suggesting that maintenance of basal levels of *Hunk* expression in the mammary glands of nulliparous mice requires ovarian hormones (Fig. 3A). Treatment of oophorectomized animals

with 17ß-estradiol alone increased *Hunk* mRNA expression but to levels below those observed in intact animals, whereas treatment with progesterone alone increased *Hunk* mRNA expression to levels comparable to those observed in intact animals. In contrast, treatment of oophorectomized animals with a combination of 17ß-estradiol and progesterone resulted in a 14-fold increase in the level of *Hunk* mRNA relative to control oophorectomized animals and a 3-fold increase relative to intact animals. The magnitude of the increase in *Hunk* expression in response to estradiol and progesterone is similar to that observed during early pregnancy. These observations suggest that the increase in *Hunk* mRNA expression observed during early pregnancy may result, either directly or indirectly, from increases in circulating levels of estradiol and progesterone.

Treatment of mice with ovarian hormones also affected *Hunk* expression in the uterus (Fig. 3B). Steady-state *Hunk* mRNA levels in oophorectomized animals increased almost two-fold relative to those in intact mice suggesting that circulating levels of ovarian hormones in nulliparous mice repress *Hunk* expression in the uterus. Treatment of oophorectomized animals with either 17ß-estradiol alone or 17ß-estradiol plus progesterone decreased *Hunk* expression to levels below those observed in both intact and oophorectomized animals. In contrast to findings in the mammary gland, progesterone treatment had little if any effect on *Hunk* expression in the uterus. These findings suggest that the increase in *Hunk* mRNA expression observed in the uterus following oophorectomy is due, either directly or indirectly, to loss of tonic inhibition of *Hunk* expression by estradiol.

The effect of hormones on *Hunk* expression in the mammary gland and uterus were confirmed by in situ hybridization performed on tissues from the same experimental animals as described above (Fig. 3D and not shown). Consistent with RNase protection results, oophorectomy resulted in a marked decrease in *Hunk* mRNA expression in the mammary epithelium and the combination of 17ß-estradiol and progesterone resulted in a synergistic increase in *Hunk* expression. Reminiscent of *Hunk* expression in early pregnancy, the upregulation of *Hunk* expression in oophorectomized animals treated with 17ß-estradiol and progesterone occurred in a subset of cells in both epithelial ducts and in developing alveolar buds.

Since the above experiments involved the chronic administration of hormones, sufficient time elapsed for significant developmental changes to occur in both the mammary glands and uteri of oophorectomized animals. As such, these experiments do not distinguish whether changes in *Hunk* expression reflect direct regulation by ovarian hormones, or are a consequence of the changes in epithelial proliferation and differentiation that occur in response to the chronic administration of these hormones.

In order to address this issue, intact mice were treated with a combination of 17ß-estradiol and progesterone for 24 hours prior to sacrifice (Fig. 3C). Mice treated in such a manner do not develop the marked morphological changes characteristic of long-term hormone administration. Analysis of *Hunk* mRNA expression levels in these mice revealed a similar pattern to that observed in mice treated chronically with these hormones. Within 24 hours following treatment with hormones, steady-state levels of *Hunk* mRNA increased in the mammary gland and decreased in the uterus. These findings suggest that the regulation of *Hunk* expression in these tissues by estradiol and progesterone is not solely a consequence of the changes in mammary gland and uterine architecture that occur in response to chronic hormone treatment, but rather may be a direct result of regulation by these hormones.

Hunk Expression in Transgenic Breast Cancer Cell Lines is Oncogene-Restricted

To begin to investigate the potential role of *Hunk* in mammary carcinogenesis, *Hunk* mRNA expression levels were analyzed in 3T3 fibroblasts, non-transformed mammary epithelial cell lines, and in cell lines derived from independent adenocarcinomas arising in transgenic mice expressing either the *neu, c-myc*, *H-ras*, or *int-2* oncogenes under the control of the MMTV LTR (Fig. 4) (Leder et al., 1986; Sinn et al., 1987; Muller et al., 1988; Muller et al., 1990; Morrison et al., 1994). All cell lines were harvested while actively proliferating as evidenced by their similar levels of histone *H2B* mRNA expression. Consistent with its epithelial expression, *Hunk* was not expressed in 3T3 fibroblasts, but was expressed at moderate levels in one of two non-transformed mammary epithelial cell lines. Surprisingly, *Hunk* was expressed at high levels in all eight cell

lines derived from mammary tumors that arose in MMTV-*neu* and MMTV-*H-ras* transgenic mice, whereas *Hunk* was not detected in any of the five cell lines derived from breast tumors that arose in MMTV-*c-myc* transgenic mice. Low levels of *Hunk* were detected in one of two MMTV-*int-2* derived cell lines.

Generation of MMTV-*Hunk* Transgenic Animals

The tightly regulated expression of *Hunk* observed in the mammary gland during pregnancy suggested the possibility that *Hunk* may play a role in mediating pregnancy-induced changes in the mammary gland. To test this hypothesis, transgenic mice overexpressing *Hunk* in a mammary-specific fashion were generated by using the MMTV LTR. Activity of the MMTV LTR is upregulated in epithelial cells during pregnancy and lactation in response to rising levels of prolactin, progesterone and glucocorticoids. Since expression of *Hunk* is normally upregulated early in pregnancy and returns to basal levels by mid-pregnancy, MMTV-driven expression of *Hunk* would be predicted to alter the normal profile of *Hunk* expression during mammary development.

A cDNA encoding the full-length Hunk protein was cloned downstream of the MMTV-LTR and injected into superovulated FVB mice. One of four founder lines, MHK3, was observed by Northern hybridization analysis to express the *Hunk* transgene in the mammary gland at high levels and was therefore studied further (Fig. 5A).

The tissue specificity of transgene expression in the MHK3 line was determined by RNase protection analysis of total RNA isolated from organs of the adult mouse (Fig. 5B). RNase protection analysis using a transgene-specific probe confirmed that MHK3 transgenic females express high levels of the *Hunk* transgene in the mammary gland, and further revealed lower but detectable levels of transgene expression in the spleen, salivary gland, lung and thymus, as has been observed for other MMTV transgenic mouse models. Thus, expression analysis confirmed that MHK3 animals overexpress *Hunk* in a mammary-specific manner.

In order to determine if *Hunk* mRNA levels in transgenic mice reflect changes in Hunk protein levels, antisera specific to Hunk were used to analyze Hunk protein levels in extracts prepared from lactating mammary glands of MHK3 transgenic and wild-type mice (Fig. 5C and (Gardner et al., submitted)). Immunoblotting of immunoprecipitated Hunk using specific antisera detected increased amounts of Hunk in extracts prepared from transgenic as compared to wild-type mammary glands (Fig. 5C). The failure to detect Hunk in wild-type mammary extracts is most likely a consequence of the fact that endogenous *Hunk* mRNA expression in the mammary gland is low during lactation, whereas MMTV-driven transgene expression is typically very high (Fig. 1).

In order to demonstrate that Hunk-associated kinase activity was also elevated in MHK3 transgenic animals, in vitro kinase assays were performed. Hunk was immunoprecipitated from protein extracts prepared from the lactating mammary glands of wild-type or transgenic mice. Control immunoprecipitation reactions were carried out in the absence of anti-Hunk antisera. The resulting immunoprecipitates were incubated with [γ-^{32}P]ATP and histone H1. As predicted based on the relative quantities of Hunk immunoprecipitated from transgenic and wild-type mammary glands, Hunk-associated phosphotransferase activity was substantially greater in immunoprecipitates prepared from transgenic as compared to wild-type mammary glands. These experiments confirm that MHK3 transgenic animals overexpress both Hunk protein and Hunk-associated phosphotransferase activity.

It was noted that some MHK3 animals bearing the appropriate MMTV-Hunk integration site did not express the transgene. Therefore, all MHK3 animals were analyzed for transgene expression and transgene integration site, and were classified as either transgene-expressing or transgene non-expressing. Transgene silencing has been observed in other MMTV transgenic models and provides a genetic control for ensuring that observed phenotypes are due to transgene expression rather than site-specific integration effects (Betzl et al., 1996; Sternlicht et al., 1999).

Mammary Development in Nulliparous MMTV-*Hunk* Mice

We initially analyzed the effect of Hunk overexpression on ductal morphogenesis in nulliparous MHK3 transgenic mice. As analyzed by whole mount and histological analysis, the rate of ductal elongation and extent of epithelial side-branching appeared grossly normal in mammary glands from 5-6 week old nulliparous transgenic mice.

In order to screen for potential differences in mammary development in MHK3 transgenic mice at the molecular level, we determined the level of expression of a panel of milk protein genes in mammary glands from 5-6 week old nulliparous wild-type, transgene expressing and non-expressing MHK3 animals (Fig. 6)(Robinson et al., 1995 and C. D'Cruz, unpublished results). Although milk protein genes are upregulated during pregnancy and are expressed at highest levels during lactation, each of these genes is also expressed at detectable levels in the mammary glands of nulliparous animals (C. D'Cruz and L. Chodosh, unpublished results). As such, the expression of these genes can be used as a molecular correlate for the level of mammary epithelial differentiation. Northern analysis revealed that mRNA expression of milk protein genes for ß-casein, κ-casein, α-lactalbumin, WDNM1 and WAP in adolescent females was unaffected by Hunk overexpression (Fig. 6 and data not shown). In contrast, steady-state levels of the milk protein gene, lactoferrin, were markedly higher in the mammary glands of transgenic animals as compared to age-matched wild-type animals or non-expressing MHK3 transgenic animals. These observations suggest that Hunk does not cause precocious differentiation in the mammary gland during puberty, but rather may specifically activate pathways resulting in lactoferrin upregulation.

Hunk Overexpression Inhibits Normal Lobuloalveolar Development

Consistent with the supposition that Hunk plays a role in mammary gland development during pregnancy, we initially noted that the number of pups successfully reared by MHK3 transgenic females was significantly reduced compared to wild-type animals, with many pups dying within 1-2 days after birth. Offspring of transgenic males mated to wild-type females

displayed survival rates comparable to those observed for offspring of wild-type crosses. These observations suggested that MHK3 females may have a defect in lactation.

In order to address this hypothesis, MHK3 transgenic females were sacrificed at different stages of pregnancy and lactation. Whole mount analysis and hematoxylin and eosin-stained sections at day 12.5 of pregnancy revealed no obvious morphological differences between wild-type and MHK3 transgenic animals (Fig 7A and B). In contrast, marked differences were observed between wild-type and transgenic animals at day 18.5 of pregnancy. Analysis of whole mounts and hematoxylin and eosin-stained sections at this stage of development consistently showed decreased lobuloalveolar development in MHK3 transgenic animals. Decreased lobuloalveolar development was also observed in MHK3 females sacrificed at day 2 of lactation. Normally during lactation the mammary gland is filled with casein-secreting lobules such that by whole-mount analysis the gland is entirely opaque, and by histological analysis of hematoxylin and eosin stained sections no white adipose tissue is seen (Fig. 7A and B). In contrast to wild-type and non-expressing MHK3 females, lobuloalveolar units in lactating Hunk-overexpressing animals are smaller and appear less developed by whole mount analysis (Fig. 7A and data not shown). Analysis of H&E stained sections revealed that only half of the mammary fat pad of lactating MHK3 mice is occupied by secretory alveoli (Fig. 7B). This defect in lactation does not appear to be due to the inability of alveolar cells to proliferate since the number of alveoli is similar in transgenic and wild-type glands (Fig. 7B). Rather, alveoli in transgenic animals are less distended with milk. These observations suggest that dysregulated expression of *Hunk* prevents terminal differentiation of the mammary gland during late pregnancy and lactation.

Hunk Overexpression Alters Milk Protein Gene Expression

Normal mammary development during pregnancy involves both proliferation and differentiation of alveolar epithelial cells. Alveolar cell proliferation occurs primarily during the first half of pregnancy, while alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. The dramatic changes which the breast undergoes during lobuloalveolar

development are reflected on a molecular level by the tightly regulated and temporally ordered expression of distinct milk protein genes during pregnancy and lactation(Robinson et al., 1995). While steady-state mRNA levels for each of these genes typically increase progressively throughout the course of pregnancy, each gene undergoes a maximal increase in expression at a characteristic time in pregnancy. This differential expression permits individual milk protein genes to be classified as early (ß-casein), intermediate (κ-casein, lactoferrin), late-intermediate (WAP) or late (ε-casein) markers of epithelial differentiation (D'Cruz, unpublished; Robinson et al., 1995). These reproducible temporal patterns of gene expression permit the identification and analysis of mammary epithelial cells at distinct steps in a differentiation pathway.

In order to further analyze the defect in lobuloalveolar development observed in MHK3 transgenic glands, mRNA expression levels of a panel of milk protein genes in mammary glands from transgenic and wild-type animals was assessed during pregnancy and lactation (Fig. 8). Although no marked morphological differences were noted in transgenic mice at day 12.5 of pregnancy, steady-state levels of expression for all five milk protein genes in mammary glands from MHK3 transgenic mice were 25% to 65% of those observed in wild-type mice (Fig. 8A and B). This suggests that mammary glands from Hunk-overexpressing transgenic mice are less differentiated compared to wild-type glands at this stage of development.

A similar analysis performed at day 18.5 of pregnancy revealed more dramatic differences between wild-type and transgenic glands, consistent with the morphological defects observed at this stage of development. Although the mRNA expression levels for all five milk proteins tested were reduced relative to wild-type controls, the 10-fold reduction in mRNA expression observed for the late differentiation marker ε-casein was considerably more pronounced than the 2 to 3-fold reduction observed for the early and intermediate differentiation markers, ß-casein, κ-casein and lactoferrin.

Finally, analysis of this same panel of molecular markers was performed for mice at day 2 of lactation (Fig. 8E and F). This analysis revealed a pattern similar to that observed late in pregnancy, in which the reductions in milk protein gene expression observed were greater for the

late differentiation marker, ε-casein, as compared to the early and intermediate markers ß-casein, κ-casein and WAP. The observation that no significant differences in milk protein gene expression were observed between wild-type mice and non-expressing transgene-positive mice strongly argues that the abnormalities in mammary gland development observed in MHK3 mice are due to expression of the MMTV-*Hunk* transgene, rather than to the insertional disruption or activation of an endogenous gene.

Surprisingly, at day 2 of lactation, lactoferrin expression was actually higher in expressing transgenic glands as compared to both wild-type and non-expressing transgenic glands. This is particularly interesting since lactoferrin is also upregulated in the mammary glands of nulliparous MHK3 animals. This observation suggests that while Hunk overexpression may have a global inhibitory effect on differentiation of the mammary gland during late pregnancy and lactation, the effects of Hunk on lactoferrin expression are distinct and may represent a specific effect of this kinase on signal transduction pathways regulating lactoferrin expression.

DISCUSSION

We previously described the cloning and initial characterization of a novel SNF1-related serine/threonine kinase, *Hunk*. In this report we demonstrate that *Hunk* is expressed in a heterogeneous pattern in the mammary epithelium, exhibits an oncogene-restricted pattern of expression in transgenic breast cancer cell lines, and is regulated in the mammmary gland during pregnancy and in response to estradiol and progesterone. Consistent with a proposed role in regulating pregnancy-induced changes in mammary development, misexpression of Hunk in the mammary gland disrupts normal lobuloalveolar development during late pregnancy and lactation. Together, these data suggest that Hunk contributes to signal transduction pathways that mediate pregnancy-induced changes in the mammary gland, and that Hunk may play a role in the response of the mammary epithelium to ovarian hormones. In addition, we demonstrate that *Hunk* is expressed in a subset of mammary epithelial cells in vivo, as well as in vitro in a subset of transgenic breast cancer cell lines. These findings suggest that *Hunk* may be expressed in a biologically interesting subset of epithelial cells in the mammary gland, and may be involved in the process of mammary carcinogenesis.

Several SNF1-related kinases have been implicated in vertebrate development on the basis of their temporal and spatial patterns of expression (Ruiz et al., 1994; Becker et al., 1996). In a similar manner, our analysis of *Hunk* expression suggests a developmental role for Hunk in the mammary gland during pregnancy. *Hunk* mRNA expression levels remain low and relatively constant throughout virgin development and then rise dramatically during early pregnancy, a period characterized by rapid alveolar cell proliferation. However, multiple lines of evidence suggest that *Hunk* expression does not correlate with proliferation. Specifically, the temporal expression pattern of *Hunk* in the mammary gland during development is distinct from that of genes which serve as markers of proliferation by virtue of their cell cycle-regulated patterns of expression. *Hunk* is not upregulated during puberty, whereas proliferation markers such as *Cyclin A*, *Plk*, *Brca1*, and *Brca2* are each upregulated during puberty as well as during early pregnancy (Marquis et al., 1995; Rajan et al., 1997). *Hunk* is also not preferentially expressed in proliferative as

compared to non-proliferative compartments in the mammary gland (i.e. terminal end buds vs, ducts during puberty, alveoli vs. ducts during early pregnancy). Finally, analysis of actively growing versus confluent mammary epithelial cells reveals no difference in *Hunk* mRNA levels (H. Gardner, unpublished). These observations suggest that *Hunk* expression does not simply reflect the proliferative state of the breast, but rather may reflect other developmental events in the mammary gland.

While the concept that the mammary epithelium is comprised of a variety of cellular subtypes is relatively established, the number of molecular markers available to identify such subtypes has remained surprisingly low (Taylor-Papadimitriou et al., 1993). The heterogeneous spatial expression pattern of *Hunk* in the mammary gland suggests that expression of this kinase may identify a subset of mammary epithelial cells. We have previously reported a heterogeneous spatial expression pattern for *Hunk* in other organs including brain, thymus, prostate, and duodenum (Gardner et al., submitted). Within each of these tissues *Hunk* expression is restricted to small subsets of cells within specific cellular compartments. It is not clear, however, whether only a subset of cells is capable of expressing *Hunk* (i.e. *Hunk* marks a cell type) or whether all cells express *Hunk* under certain conditions (i.e. *Hunk* marks a cellular state). Additional experiments will be required to distinguish between these possibilities.

Amplification and overexpression of the human homologue of *neu, ErbB2,* in human primary breast cancers is correlated with aggressive tumor phenotype and poor clinical prognosis. Moreover, increasing evidence suggests that patients with *ErbB2*-positive breast cancers respond to treatment with the chemotherapeutic agent, doxorubicin, whereas patients with *ErbB2*-negative breast cancers do not (Clark, 1998; Paik et al., 1998; Thor et al., 1998). As such, studies of the signal transduction pathways activated by neu, and of the epithelial cell types transformed by neu, should contribute to our understanding of the biological differences in tumors that arise via different oncogenic pathways.

In this context, transgenic mice in which the MMTV promoter is used to direct the mammary-specific overexpression of oncogenes such as *neu*, v-Ha-*ras*, *int-2* and c-*myc* have been widely

used as models of human breast cancer. Overexpression of each of these oncogenes results in the formation of mammary adenocarcinomas with histological patterns that are characteristic for each oncogene (Cardiff et al., 1991; Cardiff et al., 1993). Therefore, identification of the differentially expressed genes responsible for these histological differences might shed light on the signal transduction pathways leading to transformation, or on the identity of cell types that are preferentially transformed by different oncogenes (Cardiff et al., 1993; Morrison et al., 1994). As such, the mechanism by which *Hunk* is preferentially expressed in *neu* and *v-Ha-ras,* as compared to *c-myc* and *int-2,* tumor cell lines may be important for understanding distinguishing features of the transformation process initiated by different oncogenes.

The oncogene-restricted pattern of *Hunk* expression in breast cancer cell lines derived from the *neu*, v-Ha-*ras*, *int-2* and c-*myc* transgenic models suggests either that *Hunk* expression is upregulated by the neu and v-Ha-ras pathways, or that neu and v-Ha-ras preferentially transform a mammary epithelial cell type that is defined by its expression of *Hunk* (Morrison et al., 1994). This latter model predicts that *Hunk* represents a marker for a biologically important subpopulation of epithelial cells in the breast and is consistent with the heterogeneous spatial expression pattern of this kinase. Interestingly, we have identified a second protein kinase, *Punc*, that is likewise regulated in the mammary gland during pregnancy, is expressed in a heterogeneous pattern in the mammary epithelium, and exhibits an oncogene-associated pattern of expression (H. Gardner, unpublished). In contrast to *Hunk,* however, *Punc* exhibits the inverse expression pattern in transgenic breast cancer cell lines, with high expression levels in cell lines derived from MMTV-c-*myc* and MMTV-*int-2* tumors and undetectable levels in cell lines derived from MMTV-*neu* and MMTV-Ha-*ras* tumors. Furthermore, whereas *Hunk* is upregulated in the mammary gland early in pregnancy, *Punc* expression in the mammary gland peaks late in pregnancy. These findings suggest that *Hunk* and *Punc* may identify two biologically important mammary epithelial cell types, or that these kinases may represent downstream targets of oncogenes known to cause breast cancer.

Hunk upregulation in the mammary gland during early pregnancy is transient as *Hunk* expression returns to baseline levels by mid-pregnancy, and is subsequently downregulated during late pregnancy and lactation. This observation raises the intriguing possibility that the tightly regulated pattern of *Hunk* expression during pregnancy is required for the normal differentiation of the mammary epithelium during this phase of development. We have tested this hypothesis by misexpressing *Hunk* in the mammary glands of transgenic mice. Forced overexpression of an MMTV-*Hunk* transgene in the mammary epithelium throughout pregnancy and lactation results in a defect in lobuloalveolar development and lactation with molecular abnormalities first discernible by mid-pregnancy, and morphological abnormalities first discernible by late pregnancy. In contrast, forced overexpression of *Hunk* during virgin mammary development appears to have no obvious effect on the formation of the mammary epithelial tree. Together, these observations suggest that high levels of Hunk expression are not sufficient to disrupt ductal morphogenesis during puberty, yet are sufficient to disrupt normal lobuloalveolar development during pregnancy. This, in turn, suggests that the downregulation of *Hunk* that normally occurs after early pregnancy may be required for normal lobuloalveolar development to proceed.

Surprisingly, lactoferrin expression in the mammary glands of nulliparous and lactating mice was elevated in Hunk-overexpressing MHK3 animals as compared to non-expressing transgenic and wild-type animals. This observation suggests that while Hunk may have a negative effect on the global differentiation of the mammary gland during late pregnancy and lactation, the effects of Hunk on lactoferrin expression may be more specific. In confirmation of this hypothesis, recent data from oligonucleotide-based cDNA mircroarrays comparing gene expression patterns in wild-type and MHK3 virgin transgenic glands indicate that lactoferrin is one of only 16 genes of the approximately 6350 genes analyzed whose expression changes by more than 2.5-fold (H. Gardner, unpublished results). These results suggest that the effects of aberrant *Hunk* expression in the mammary gland are limited to a discrete number of pathways, at least one of which results in lactoferrin upregulation. Since lactoferrin expression in different tissues is known to be regulated by 17ß-estradiol, EGF, Protein Kinase C and cAMP-mediated pathways (Teng,

1995), the specific upregulation of lactoferrin expression in MHK3 transgenic mice may provide clues to signal transduction pathways in which Hunk is involved.

We have demonstrated that treatment of mice with 17ß-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in the mammary gland. These findings suggest that the upregulation of *Hunk* expression in response to hormones is not a consequence of the marked changes in epithelial proliferation and differentiation that occur either during early pregnancy or in response to the chronic administration of 17ß-estradiol and progesterone. At present, however, we cannot distinguish whether changes in *Hunk* expression reflect direct or indirect regulation by these hormones.

Since steroid hormone receptors can be activated in a ligand-independent manner by diverse signaling pathways, and since steroid hormone receptor function can be modulated by phosphorylation, our findings raise the possibility that the upregulation of *Hunk* expression by estradiol and progesterone may contribute to changes in the mammary epithelium that occur during pregnancy. In this context, it is plausible to hypothesize that Hunk is involved in mediating the effects of steroid hormones, or in modulating the function of the receptors themselves. As such, elucidating the mechanisms by which hormones regulate *Hunk* expression levels, and by which Hunk may regulate mammary epithelial proliferation or differentiation, may yield insights into the complex role which hormones play in mammary development and carcinogenesis.

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FIGURE LEGENDS

Figure 1. Temporal regulation of *Hunk* expression during mammary gland development. A) RNase protection analysis of *Hunk* mRNA expression during postnatal developmental of the murine mammary gland. 40 μg of total RNA isolated from mammary glands at the indicated timepoints was hybridized to a ^{32}P-labeled antisense RNA probe specific for *Hunk*. A ^{32}P-labeled antisense RNA probe specific for ß-*actin* was included in the same hybridization reaction as an internal loading control. B) Phosphorimager analysis of RNase protection analysis in A. *Hunk* expression was quantitated and normalized to ß-*actin* expression to correct for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels are shown relative to adult virgin (15 wk). C) In situ hybridization analysis of *Hunk* expression during pregnancy and lactation. Bright-field (top panel) and dark-field (bottom panel) photomicrographs of mammary gland sections from day 7 pregnant, day 20 pregnant or day 9 lactating animals hybridized with an ^{35}S-labeled *Hunk*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were identical for all dark field photomicrographs to illustrate changes in *Hunk* expression during pregnancy. d, duct; al, alveoli; st, adipose stroma. 300x magnification.

Figure 2. Heterogeneous expression of *Hunk* in the mammary epithelium. In situ hybridization analysis of *Hunk* expression in the virgin mammary gland using an ^{35}S-labeled *Hunk*-specific antisense probe. Bright-field (A-C) and dark-field (D-F) photomicrographs of in situ hybridization analysis performed on mammary gland sections from 5 week-old nulliparous females. In all cases note the heterogeneous expression pattern and similar expression levels of *Hunk* in both epithelial ducts (A, C, D, and F) and terminal end buds (B, C, E, and F). No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were optimized for each dark field panel. d, duct; eb, terminal end bud. 750x magnification.

Figure 3. Ovarian hormones alter *Hunk* expression in vivo. RNase protection analysis of *Hunk* mRNA expression in mammary glands and uteri of mice treated with ovarian hormones. Tissues were harvested from either intact females (sham) or oophorectomized females that received daily subcutaneous injections of either vehicle alone (OVX), 17β-estradiol (OVX+E_2), progesterone (OVX+P), or both 17β-estradiol and progesterone (OVX+E_2+P) for fourteen days. Each sample represents a pool of at least 10 mice. 20 μg of total RNA isolated from the mammary glands (A) or uteri (B) of treated animals was hybridized overnight with ^{32}P-labeled antisense RNA probes specific for *Hunk* and ß-*actin*. Signal intensities were quantitated by phosphorimager analysis and *Hunk* expression was normalized to *β-actin* expression levels. *Hunk* expression relative to expression in oophorectomized (OVX) controls is shown below each lane. C) Quantitation of *Hunk* expression in mammary glands and uteri from intact FVB female mice sacrificed 24 hours following injection with PBS (control; light shaded boxes) or a combination of 5 mg progesterone in 5% gum arabic and 20μg of β-estradiol in PBS (+E_2+P; dark shaded boxes). RNase protection analysis was performed on either 20 μg (breast) or 40 μg (uterus) of total RNA using ^{32}P-labeled antisense RNA probes specific for *Hunk* and ß-*actin*. *Hunk* expression was quantitated by phosphorimager analysis, normalized to β-actin, and values are shown relative to control animals. Each bar represents the average of four individually processed animals ± SE for each group. D) In situ hybridization analysis of *Hunk* expression in mammary gland sections from oophorectomized mice treated with hormones as described in (A). Dark field exposure times were identical in all cases. d, duct; al, alveoli; st, adipose stroma. 300x magnification.

Figure 4. *Hunk* expression in transformed and non-transformed murine cell lines. Transformed cell lines were derived from mammary adenocarcinomas arising in transgenic animals expressing the *int-2*, c-*myc*, *neu* or v-Ha-*ras* oncogenes in the mammary gland, as indicated. A) Northern hybridization analysis of 6 μg of poly(A)+ RNA from actively growing

murine cell lines hybridized with cDNA probes specific for *Hunk* or histone *H2B*. Cell lines are: NIH-3T3 fibroblast; non-transformed (Non-Tx): NMuMG, HC11, and CL-S1; MMTV-*int-2*: HBI2 and 1128; MMTV-c-*myc*: 8Ma1a, MBp6, M1011, M158, and 16MB9a; MMTV-*neu*: SMF, NAF, NF639, NF11005, and NK-2; MMTV-Ha-*ras*: AC816, AC711, and AC236.

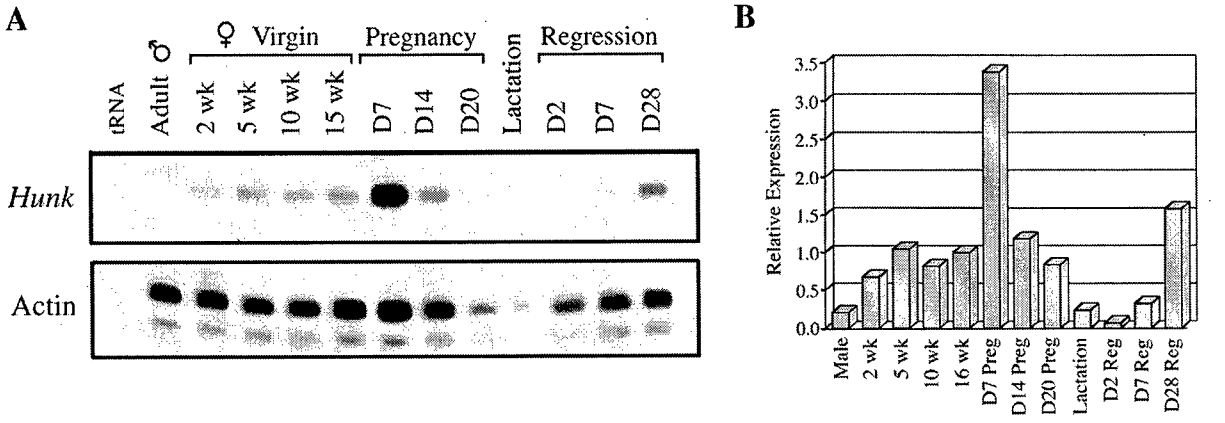
Figure 5. MMTV-*Hunk* transgene expression in MHK3 transgenic mice. The MMTV LTR was used to generate transgenic mice misexpressing *Hunk* in a mammary-specific manner. A) Northern hybridization analysis of MMTV-*Hunk* transgene expression in mammary glands from either wild-type or MHK3 transgenic mice using a ^{32}P-labeled probe specific for *Hunk*. The detected mRNA transcript corresponds to the expected size of the transgene. B) RNase protection analysis of MMTV-*Hunk* transgene expression in organs from an MHK3 transgenic female mouse. A ^{32}P-labeled antisense RNA probe spanning the junction of the 3' end of the *Hunk* cDNA and the 5' end of the SV40 polyadenylation signal was used to detect transgene expression specifically. A ^{32}P-labeled antisense RNA probe to β-*actin* was used in the same reaction to control for RNA loading and sample processing. C) Immunoprecipitation of Hunk from MHK3 transgenic animals. Affinity-purified antisera raised against the carboxyl-terminus of Hunk (IP: α-Hunk) were incubated with 500 μg of protein extract prepared from mammary glands harvested from either MHK3 transgenic (Tg) or wild-type (Wt) mice. A control reaction was performed without antisera (IP: no Ab). Immunoprecipitated protein was resolved on a 10% SDS-PAGE gel and analyzed by immunoblotting using carboxyl-terminal anti-Hunk antisera. The expected migration of Hunk is indicated. D) In vitro kinase assay of anti-Hunk immunoprecipitates. Histone H1 was used as an in vitro kinase substrate for protein immunoprecipitated with (+) or without (-) anti-Hunk antisera from extracts containing equal amounts of protein as in (C). Immunoprecipitates were incubated with [γ-32P] ATP and histone H1, separated on a 15% SDS-PAGE gel and subject to autoradiography. The relative migration of histone H1 is indicated.

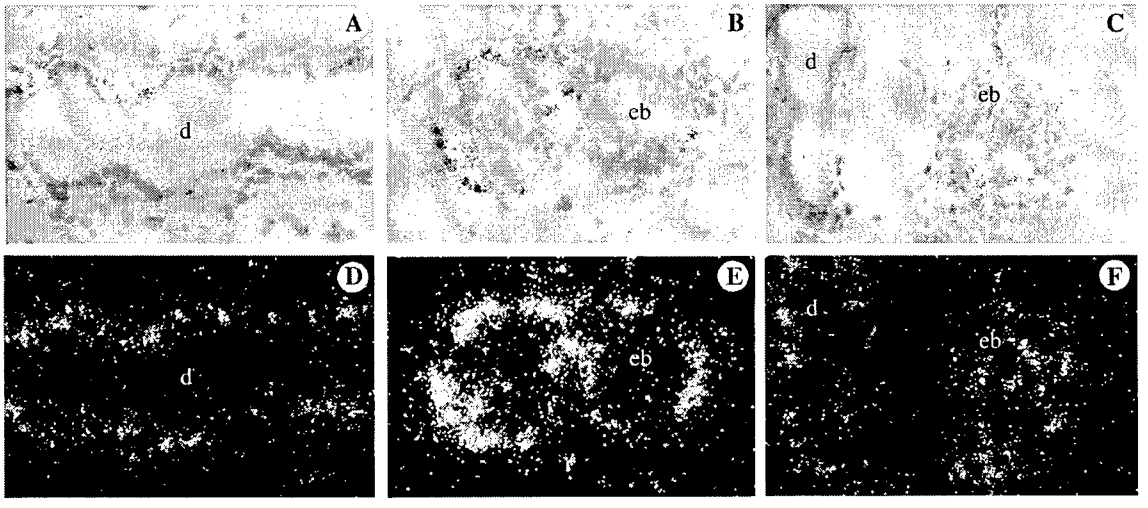
Figure 6. Upregulation of lactoferrin expression in nulliparous MHK3 mammary glands. Analysis of differentiation marker expression in mammary glands from either wild-type (light-shaded boxes), transgene-expressing (TG-expr; dark-shaded boxes), or non-expressing transgenic (TG non-expr; hatched boxes) virgin female MHK3 mice during puberty. Sample sizes were 16, 10, and 8 respectively. Northern hybridization analysis and quantitation was performed on 3 μg of total RNA isolated from mammary glands using probes specific to milk protein genes. Milk protein gene expression was normalized to *β-actin* . Wild-type expression values were set to 1.0 and values are shown as a mean ± SE for each group.

Figure 7. Morphologic defects in MHK3 transgenic mice during late pregnancy and lactation. Number four mammary glands from MHK3 transgenic and wild-type females were harvested at day 12.5 and day 18.5 of pregnancy, as well as at day 2 of lactation. At least three transgene-expressing mice and three wild-type mice were analyzed for each timepoint. A representative photomicrograph is shown for each group. A) Whole mount analysis of transgenic and wild-type mammary glands at the indicated timepoints. Harvested glands were fixed and stained with Carmine dye in order to visualize epithelial ducts and alveoli. B) Representative hematoxalin and eosin-stained sections of paraffin-embedded transgenic and wild-type mammary glands. al, alveoli; lo, lobule; st, adipose stroma. Magnification: (A): 6.3x (day 2 lactation); 32x (day 18.5 pregnancy); 50x (day 12.5 pregnancy); (B): 200x.

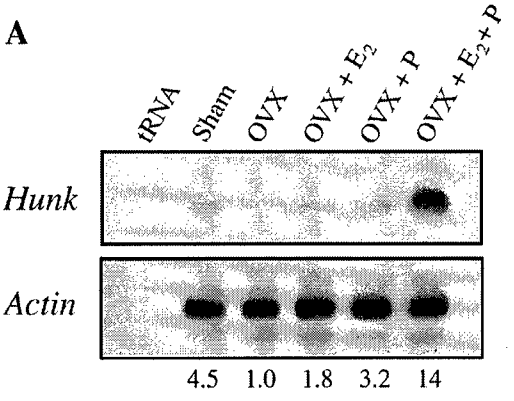
Figure 8. Molecular defects in MHK3 transgenic mice during pregnancy and lactation. A, C, E) Northern analysis using milk protein gene-specific probes and 5 μg of total RNA isolated from wild-type, MHK3 transgene-expressing, or non-expressing transgenic mammary glands at day 12.5 of pregnancy, day 18.5 of pregnancy, or at day 2 of lactation. At least three independently processed samples were analyzed from each group. The 28S ribosomal RNA band is shown as a loading control. B, D, F) Phosphorimager quantitation of Northern analysis in A, C and E. Milk protein gene expression was normalized to *β-actin* . Values for

transgene expressing mice (dark-shaded boxes) and non-expressing transgenic mice (hatched boxes) are shown normalized to wild-type expression values (light-shaded boxes) which were set to 1.0, and values are shown as a mean ± SE for each group.

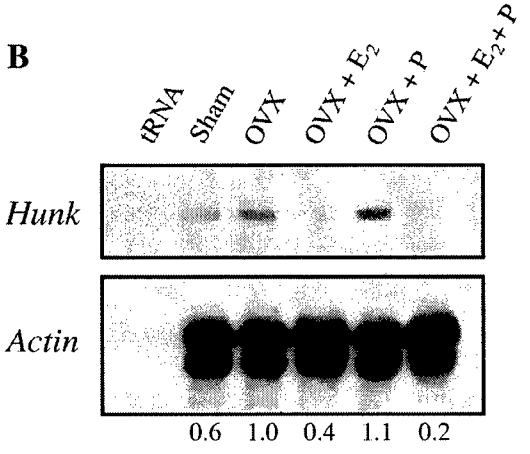

A
tRNA
Adult ♂
♀ Virgin
2 wk
5 wk
10 wk
15 wk
Pregnancy
D7
D14
D20
Lactation
Regression
D2
D7
D28
Hunk
Actin
B
Relative Expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Male
2 wk
5 wk
10 wk
16 wk
D7 Preg
D14 Preg
D20 Preg
Lactation
D2 Reg
D7 Reg
D28 Reg
C
Day 7 Pregnancy
Day 20 Pregnancy
Day 9 Lactation
st
al
d
lo


A
d
B
eb
C
d
eb
D
d
E
eb
F
d
eb

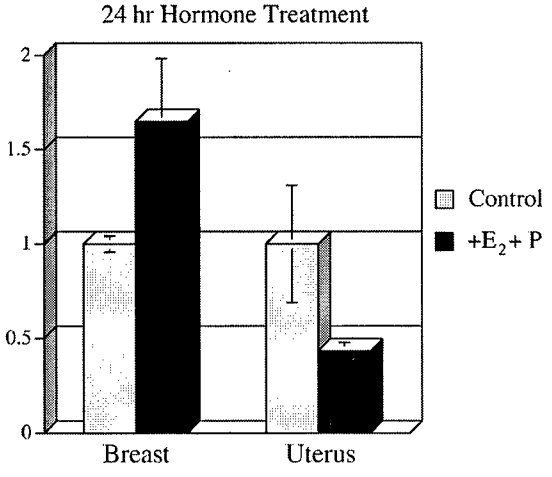
A

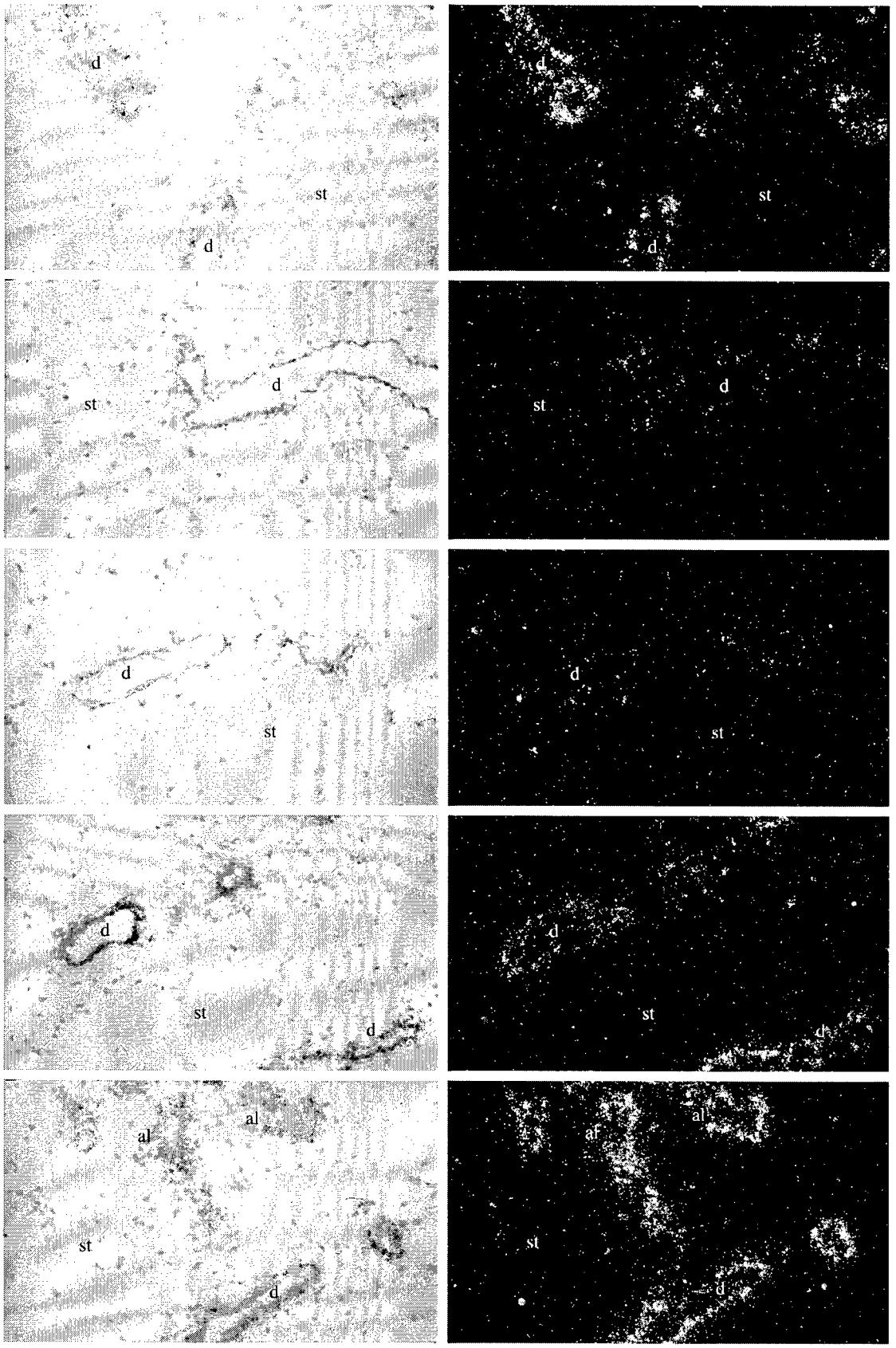

tRNA
Sham
OVX
OVX + E_2
OVX + P
OVX + E_2 + P
Hunk
Actin
4.5 1.0 1.8 3.2 14

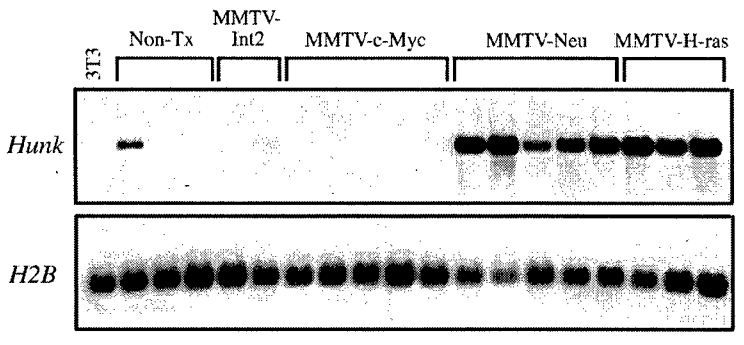
B

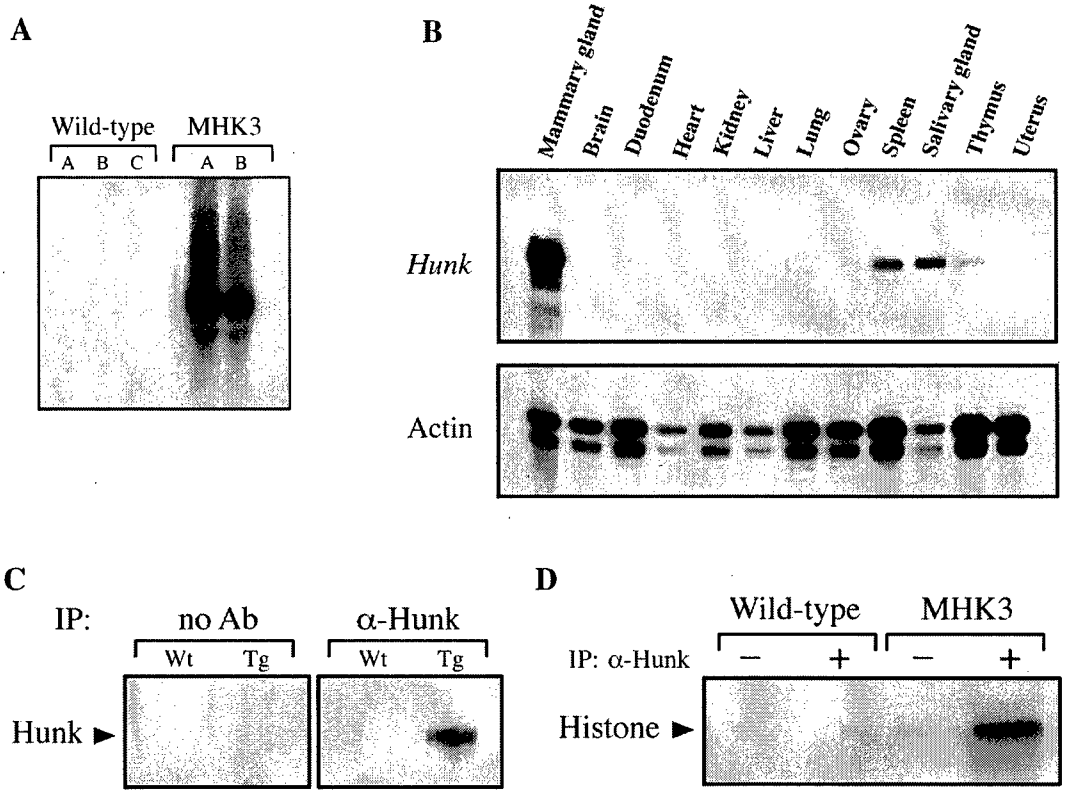

tRNA
Sham
OVX
OVX + E_2
OVX + P
OVX + E_2 + P
Hunk
Actin
0.6 1.0 0.4 1.1 0.2

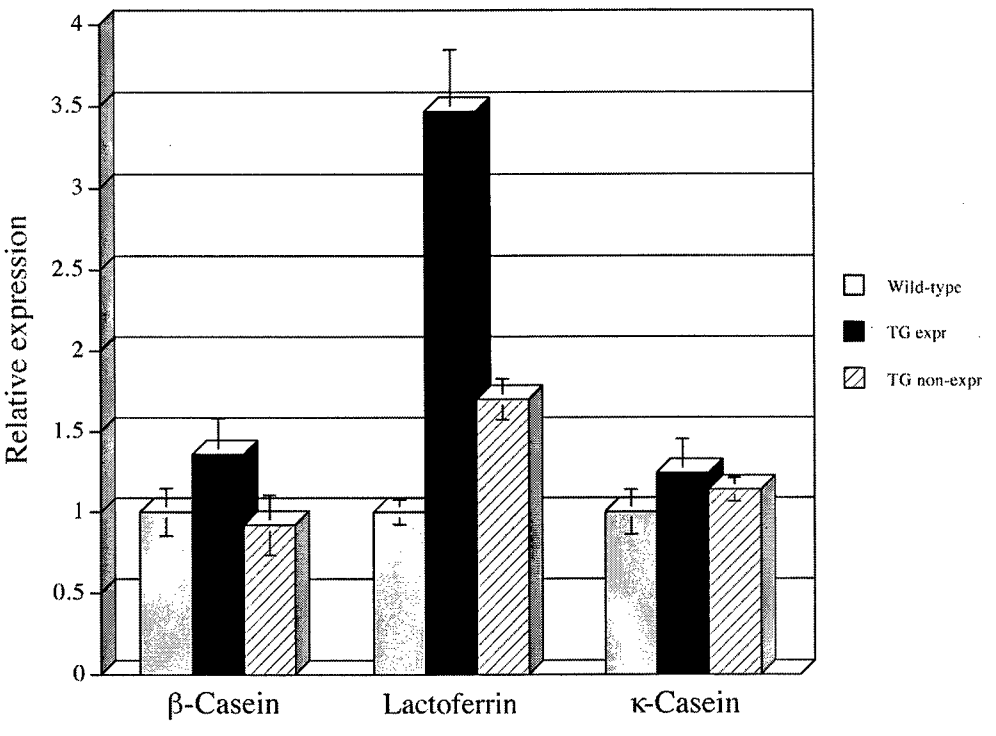
C

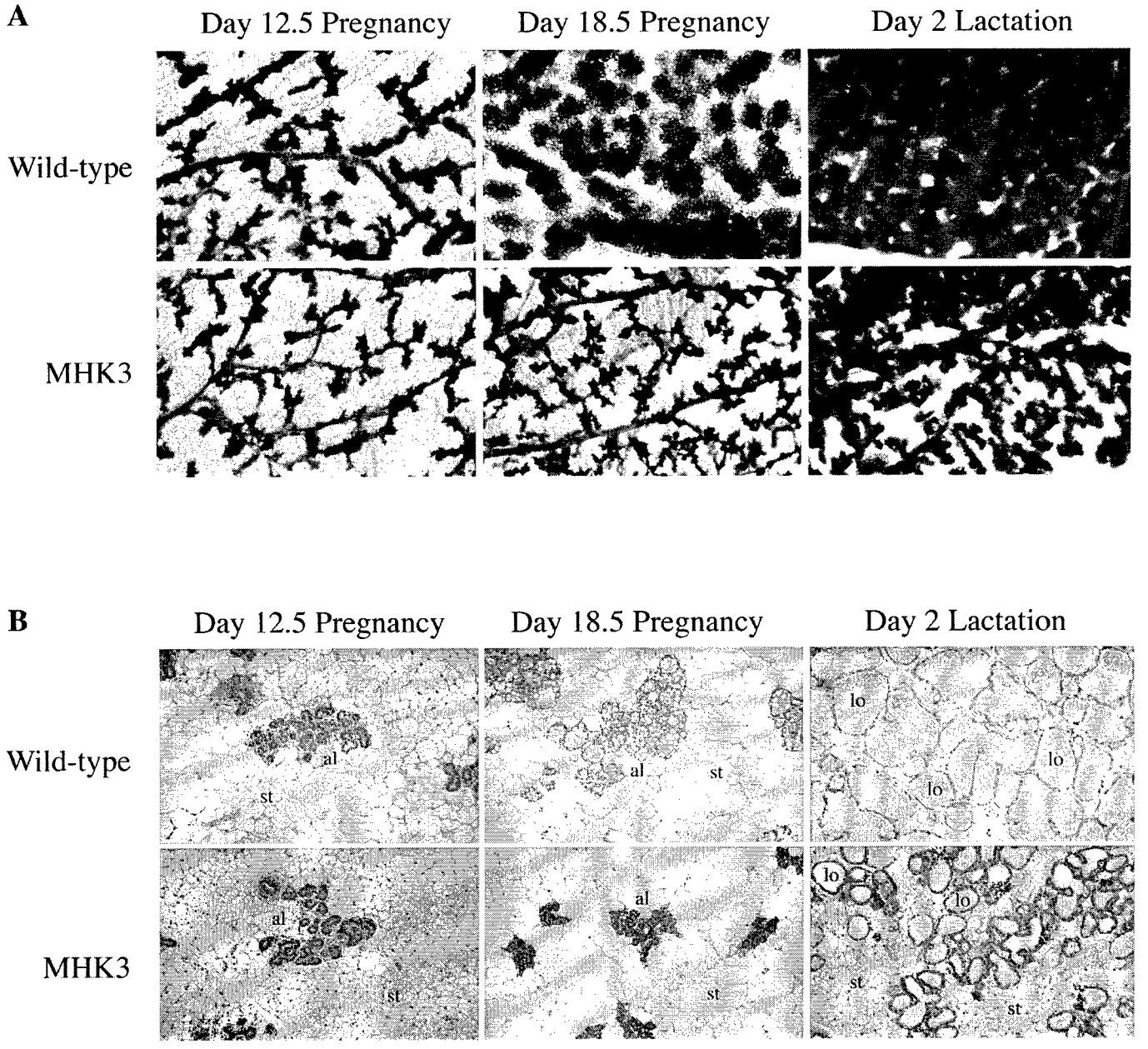

24 hr Hormone Treatment
2
1.5
1
0.5
0
Control
+E_2 + P
Breast
Uterus

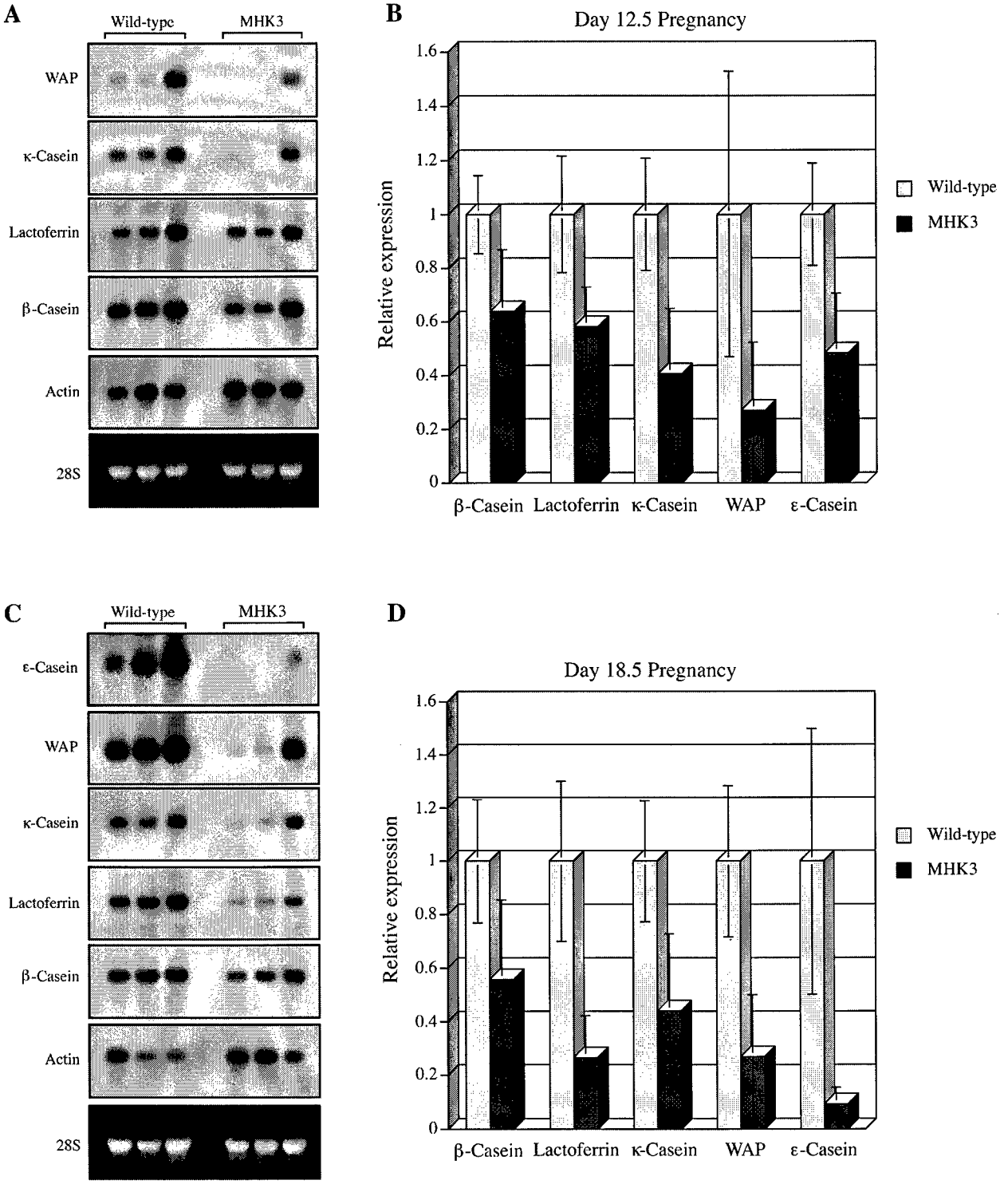

d
st
d
d
st
d
Sham
Control
st
d
d
st
d
OVX
Control
d
st
d
d
st
OVX + E_2
d
st
d
d
st
d
OVX + P
al
al
st
d
al
al
st
d
OVX
+E_2+ P


3T3
Non-Tx
MMTV-Int2
MMTV-c-Myc
MMTV-Neu
MMTV-H-ras
Hunk
H2B

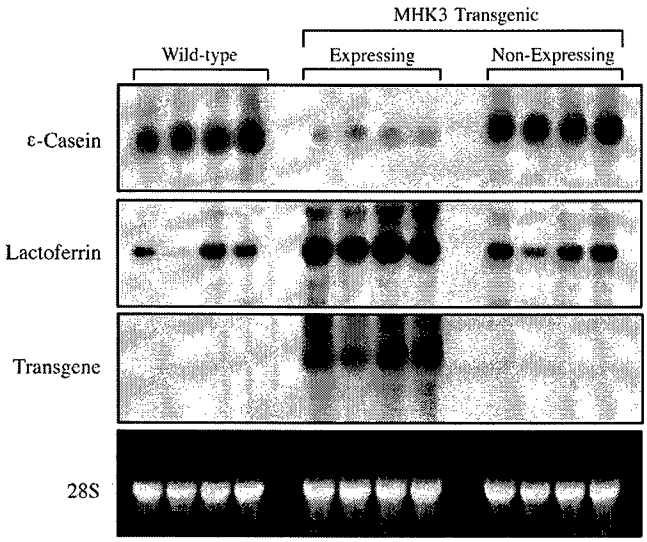

A
Wild-type
MHK3
A B C
A B
B
Mammary gland
Brain
Duodenum
Heart
Kidney
Liver
Lung
Ovary
Spleen
Salivary gland
Thymus
Uterus
Hunk
Actin
C
IP:
no Ab
α-Hunk
Wt
Tg
Wt
Tg
Hunk ►
D
Wild-type
MHK3
IP: α-Hunk
–
+
–
+
Histone ►


Relative expression
4
3.5
3
2.5
2
1.5
1
0.5
0
β-Casein
Lactoferrin
κ-Casein
Wild-type
TG expr
TG non-expr

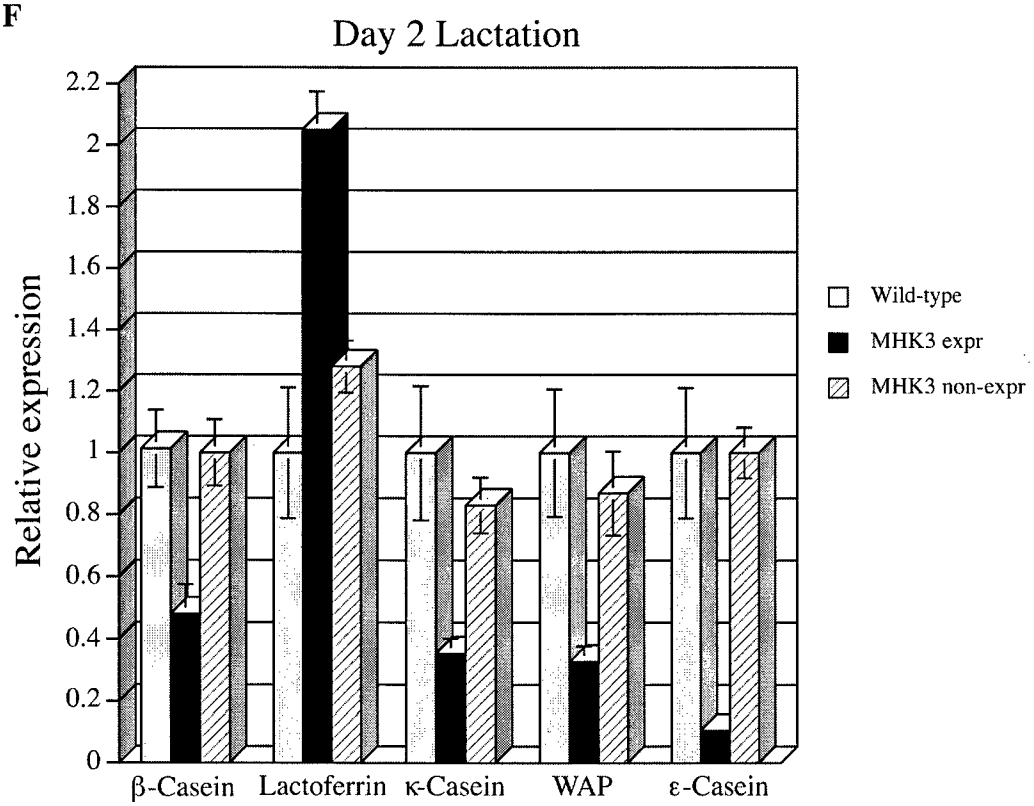

A
Day 12.5 Pregnancy
Day 18.5 Pregnancy
Day 2 Lactation
Wild-type
MHK3
B
Day 12.5 Pregnancy
Day 18.5 Pregnancy
Day 2 Lactation
Wild-type
MHK3
al
st
lo


A
Wild-type
MHK3
WAP
κ-Casein
Lactoferrin
β-Casein
Actin
28S
B
Day 12.5 Pregnancy
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
β-Casein
Lactoferrin
κ-Casein
WAP
ε-Casein
Wild-type
MHK3
C
Wild-type
MHK3
ε-Casein
WAP
κ-Casein
Lactoferrin
β-Casein
Actin
28S
D
Day 18.5 Pregnancy
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
β-Casein
Lactoferrin
κ-Casein
WAP
ε-Casein
Wild-type
MHK3

E


MHK3 Transgenic
Wild-type
Expressing
Non-Expressing
ε-Casein
Lactoferrin
Transgene
28S

F


Day 2 Lactation
Relative expression
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
β-Casein
Lactoferrin
κ-Casein
WAP
ε-Casein
Wild-type
MHK3 expr
MHK3 non-expr

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

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DAMD17-96-1-6112	ADB233138
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